Antioxidant Activity of *Centaurium erythraea* Infusion Evidenced by Its Superoxide Radical Scavenging and Xanthine Oxidase Inhibitory Activity

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Centaurium erythraea Rafin. (Gentianaceae) has long been used in traditional medicine. This plant contains considerable amounts of polyphenolic compounds, namely, xanthones and phenolic acids as the main constituents. Because phenolic groups exhibit activity as radical scavengers and/or metal chelators, this study evaluated the superoxide radical scavenging properties of a lyophilized infusion obtained from *C. erythraea* flowering tops. Superoxide radical scavenging activity was assayed using enzymatic (xanthine/xanthine oxidase) and nonenzymatic (NADH/phenazine methosulfate) superoxide generating systems. This study provided evidence that *C. erythraea* exhibits interesting antioxidant properties, expressed either by the capacity to scavenge superoxide radical or to noncompetitively inhibit xanthine oxidase. The main phenolic compounds present in this extract were several esters of hydroxycinnamic acids, namely, *p*-coumaric, ferulic, and sinapic acids.

Keywords: *Centaurium erythraea; antioxidants; polyphenols; superoxide radical scavenging activity; xanthine oxidase inhibition*

INTRODUCTION

Small centaury (*Centaurium erythraea* Rafin.) (Gentianaceae) is a medicinal plant with a long tradition, being included in the pharmacopoeias of many European and American countries. Phytochemically it is characterized by the presence of terpenoids (*1*), xanthones (*2*, *3*), and phenolic acids and their derivatives (*1*, *4*). It has been used in human traditional medicine as a digestive, stomachic, tonic, depurative, sedative, and antipyretic (*1*). The anti-inflammatory and antipyretic effects of an aqueous extract of the plant have already been observed experimentally in rats (*5*). The ethanolic extracts from dried plant material also displayed markedly antimutagenic properties in *Salmonella typhimurium* strains TA98 and TA100 (Ames assay) when tested against 2-nitrofluorene and 2-aminoanthracene (*2*). On the other hand, as far as we know, nothing has been reported on the antioxidant properties of the plant.

Aerobic metabolism entails the production of reactive oxygen species; hence, there is a continuous requirement of antioxidants for their inactivation. In various diseases, the steady state of pro-oxidants and antioxidants may be disrupted in favor of the former, leading to oxidative stress, which may affect all types of biological molecules, including DNA, lipids, proteins, and carbohydrates (*6*, *7*). Thus, oxidative stress may be involved in processes such as mutagenesis, carcinogenesis, lipid peroxidation, oxidation, and fragmentation of proteins, as well as carbohydrate damage (*8*, *9*). The protection of the organism against oxidative stress relies not only on endogenous antioxidants but also in exogenous compounds taken in food and beverages (*9*, *10*). In this respect, among numerous molecules of plant origin, flavonoids and phenolic acids have already been studied for their free radical scavenging and antioxidant properties (*11*−*14*).

Because some of the therapeutic actions of herbal drugs, such as antimutagenicity, anticarcinogenicity, and antiaging, may be due to the antioxidant activity of their constituents (*15*, *16*), it seemed to be important to evaluate the superoxide radical scavenging activity of a *C. erythraea* flowering tops infusion, because this is the common form of usage of the plant. This paper reports the scavenging effect of the lyophilized infusion on superoxide radical employing either a xanthine/xanthine oxidase (X/XO) system or an NADH/phenazine methosulfate system, as well as the effect on xanthine oxidase activity.

A preliminary evaluation of the phenolic composition of the infusion is also 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), and kaempferol were obtained from Sigma Chemical Co. (St. Louis, MO). *p*-Coumaric, ferulic, and sinapic acids were obtained from Extrasynthèse S.A. (Genay, France). All other reagents were of analytical grade. Ultrapure Milli Q water was used throughout.

Plant Material. Flowering tops of *C. erythraea* Rafin. were collected in July 1998, in the region of Penacova (central Portugal) and dried at room temperature. A voucher specimen has been deposited at the Department of Pharmacognosy of the Faculty of Pharmacy, University of Porto, Portugal.

Plant Infusion Preparation. Dried flowering tops (5 g) were extracted for 15 min with boiling water (500 mL) (*17*). The mixture was filtered over a Büchner, and the resulting

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infusion was freeze-dried (Modulyo 4K Freeze-Dryer Edwards). The yield of the lyophilized aqueous extract was 0.67 g.

Alkaline Hydrolysis of the Infusion. Ten milliliters of 2 N NaOH was added to 0.1 g of lyophilized extract. The solution was kept in the dark for 4 h, acidified with HCl, and passed through a C18 Bond Elut, preconditioned with methanol and 2 N HCl. The phenolic compounds were eluted with MeOH. This solution was taken to dryness under reduced pressure (30 °C) and dissolved in 1 mL of MeOH, and 20 μL was analyzed by HPLC.

HPLC Analysis of Phenolic Compounds. Separation of phenolic compounds was accomplished with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (5 μm particle size; 25.0 × 0.46 cm) column and a gradient of water/formic acid (19:1) (A) and methanol (B). The gradient was as follows: 0 min, 30% B; 15 min, 30% B; 20 min, 40% B; 30 min, 45% B; 50 min, 60% B; 51 min, 100% B; 58 min, 100% B; 61 min, 30% B. Elution was performed at a solvent flow rate of 1 mL/min. Detection was achieved with a diode array detector. The compounds were identified by comparing their retention times and UV−vis spectra in the 200−400 nm range with a library of spectra previously compiled by the authors. Peak purity was checked by means of the Gilson 160 SpectraViewer software contrast facilities.

Evaluation of Superoxide Radical Scavenging Activity. Antiradical activity was determined spectrophotometrically in a 96 well plate reader (Ceres 900) by monitoring the effect of the *C. erythraea* lyophilized infusion on the reduction of NBT to the blue chromogen formazan by $O_2^{\bullet -}$, at 560 nm. The superoxide production was confirmed 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 (*18*, *19*). The reaction mixtures in the sample wells consisted of NADH (166 μM), NBT (43 μM), lyophilized infusion (26.0, 52.1, 104.2, 208.3, 416.7, and 833.3 μg/mL), and PMS (2.7 μM), in a final volume of 300 μL. All components were dissolved in 19 mM phosphate buffer, pH 7.4. Ferulic and *p*-coumaric acids and kaempferol were dissolved in DMSO. The reaction was conducted at room temperature for 2 min and initiated by the addition of PMS.

Enzymatic Assay. Superoxide radicals were generated by the X/XO system following a described procedure (*18*, *19*). The reaction mixtures in the sample wells consisted of xanthine (44 μM), XO (0.29 unit/mL), NBT (50 μM), and lyophilized infusion (5.2, 10.4, 20.8, 41.7, 83.3, and 166.7 μg/mL), in a final volume of 300 μL. Xanthine was dissolved in 1 μM NaOH, xanthine oxidase in 0.1 mM EDTA, and the other components in 50 mM phosphate buffer with 0.1 mM EDTA, pH 7.8. Kaempferol was dissolved in DMSO. The reaction was conducted at room temperature for 2 min and initiated by the addition of XO.

Effect on XO 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 (Shimadzu 2600), 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.

Additionally, this procedure was repeated with several concentrations of xanthine (11, 22, 44, and 88 μM) and 73.2 μg/mL of lyophilized infusion, to get the inhibitory pattern of the extract.

RESULTS AND DISCUSSION

In the present work, an effective antioxidant activity was found in *C. erythraea* flowering tops lyophilized infusion, which exhibited $O_2^{\bullet -}$ scavenging activity using the X/XO system (Figure 1A), with an IC_{50} at 22.8 μg/mL. When this method is used, the effect of the extract on the XO activity must be checked, because an inhibitory effect on the enzyme itself would also lead to a decrease of NBT reduction (*10*). In this regard we evaluated the effect of the extract on the XO activity by the metabolic conversion of xanthine to uric acid (Figure 1B). Taking into account that the extract presents XO inhibitory activity (IC_{50} = 73.2 μg/mL), it was not possible to show a clear-cut scavenging effect on $O_2^{\bullet -}$. To evaluate the inhibitory mechanism of the lyophilized infusion, its activity was tested at different substrate concentrations (Figure 2). In the presence of the extract both V_{max} and k_m were lowered, from 9.4 to 3.8 μM/min and from 26.6 to 14.6, respectively, suggesting a mixed noncompetitive inhibitory effect; that is, the constituents of the extract bind both to the enzyme and to the X/XO complex, but with grater affinity for the latter (*20*). In view of clarification, we

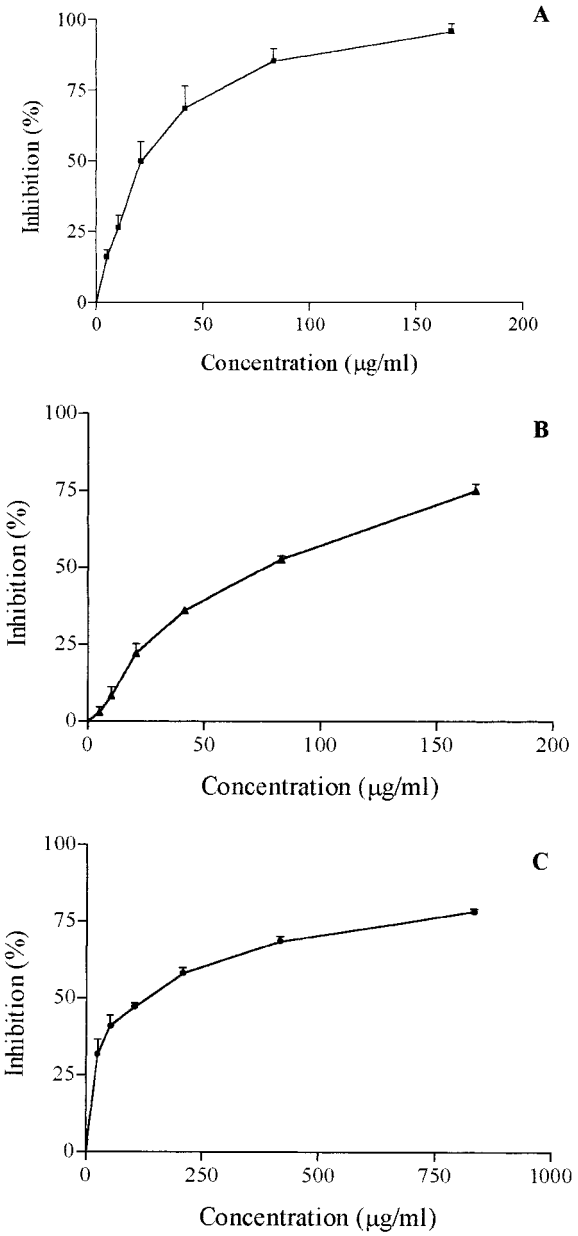


Figure 1. Effect of *C. erythraea* lyophilized infusion on (A) NBT reduction induced by superoxide radical generated in an X/XO system, (B) XO activity, and (C) NBT reduction induced by superoxide radical generated in an NADH/PMS system. Values show mean ± SE from four experiments performed in triplicate.

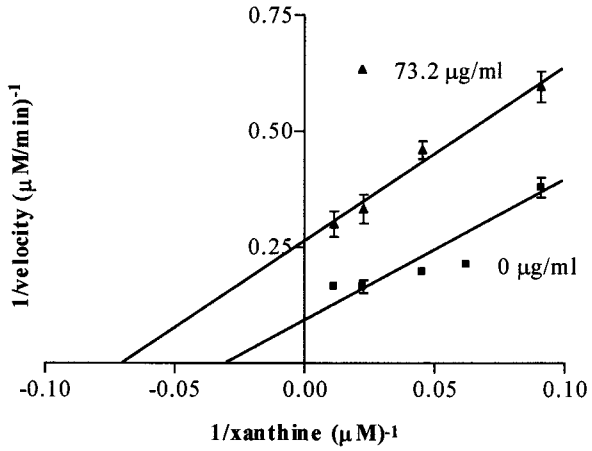


Figure 2. Lineweaver–Burk plots for the inhibition of XO by *C. erythraea* lyophilized infusion with xanthine as substrate. Values show mean ± SE from three experiments performed in triplicate.

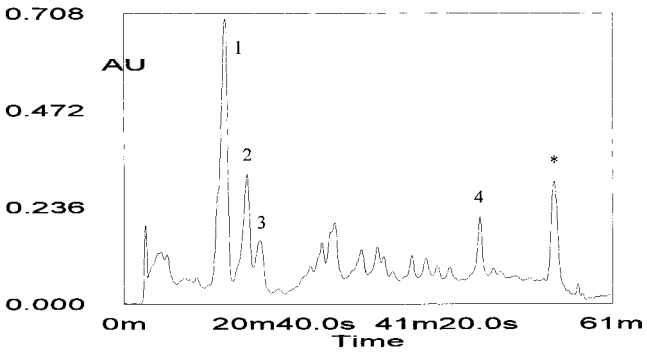


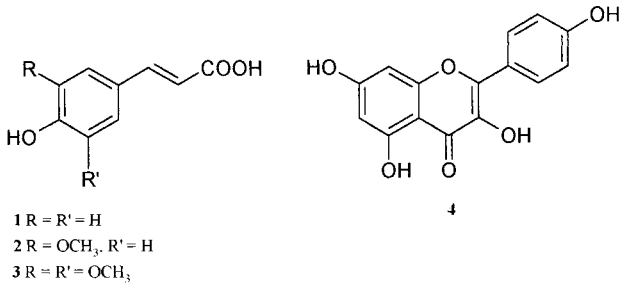
Figure 3. HPLC profile of *C. erythraea* lyophilized infusion after alkaline hydrolysis. Detection was at 320 nm. Peaks: (1) *p*-coumaric acid; (2) ferulic acid; (3) sinapic acid; (4) kaempferol; (*) unidentified xanthone.

also determined the effect of the extract on superoxide generated by the NADH/PMS system (Figure 1C), which indicated an IC_{50} of 120.2 μg/mL. Considering the results obtained, it may be anticipated that *C. erythraea* lyophilized water extract has antioxidant activity, achieved by the scavenging of superoxide radical and XO inhibition.

To try to correlate the observed antioxidant activity with the constituents of the infusion, an aliquot of the lyophilized extract was subjected to HPLC-DAD analysis. The chromatogram obtained was a very complex one, revealing chiefly substances with cinnamic acid type UV spectra. This is in good agreement with data already published referring to, in this species, the existence of bound hydroxycinnamic acids (*4*).

Once none of the compounds had a retention time or UV spectrum identical to any of the standards available and in order to find an approach to the chemical identity of the compounds, we decided to subject the extract to an alkaline hydrolysis. The hydrolyzed extract revealed mainly the presence of *p*-coumaric acid, ferulic acid, sinapic acid, kaempferol, and an unidentified xanthone (Figure 3). Other unidentified compounds were also observed.

The antioxidant activity of ferulic acid and its esters has already been evaluated (*21*, *22*), as well as that of *p*-coumaric acid and kaempferol (*22*, *23*) and is related to their conjugated rings and hydroxyl groups (*21*, *23*). However, we decided to evaluate the antioxidant activity of these compounds in the conditions used for the



1 R = R' = H
2 R = OCH_3, R' = H
3 R = R' = OCH_3

Figure 4. Structures of the identified phenolic compounds in *C. erythraea* lyophilized infusion after alkaline hydrolysis: (1) *p*-coumaric acid; (2) ferulic acid; (3) sinapic acid; (4) kaempferol.

Table 1. IC_{50} Values (Micromolar) for Tested Compounds Obtained in the in Vitro Assays

compound	nonenzymatic assay	enzymatic assay	effect on XO activity
p-coumaric acid	4668.9 ± 277.07	893.2 ± 119.91	144.9 ± 4.98
ferulic acid	2945.7 ± 320.70	587.7 ± 32.16	>50
sinapic acid	979.2 ± 30.60	70.7 ± 3.60	147.4 ± 12.10
kaempferol	116.7 ± 21.53	12.2 ± 2.06	8.6 ± 3.63

infusion. Under the tested conditions kaempferol exhibited a higher antioxidant activity than the other compounds, mainly due to its great capacity for XO inhibition, although an antiradical activity was also observed (Table 1). The phenolic acids exhibited both superoxide scavenger activity and an inhibitory effect on XO. In this case, sinapic acid revealed to be the most active superoxide scavenger, although its effect on XO was similar to that of *p*-coumaric acid. The measurement of ferulic acid XO inhibitory activity was possible only for concentrations <50 μM due to overlapping absorbances of this compound and uric acid at 295 nm.

The superoxide scavenger and inhibitory XO activities found for these compounds may be expected to contribute to the final antioxidant activity observed for the plant infusion, although it must be taken into account that they are esterified in the assayed lyophilized extract. Nevertheless, it is important to mention that the esterification does not prevent the antioxidant activity of the phenolic groups of the compounds evaluated in the present study and that the nature of the molecule bound to the phenolic acid is usually secondary (*22*, *24*).

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