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In vitro and ex vivo anti- and prooxidant components of *Cichorium intybus*

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Abstract

The water soluble antioxidant properties of *Cichorium intybus* var. *Silvestre*, whose production zone is around Chioggia, Italy, were investigated. Vegetable juices were obtained by centrifugation, and (1) filtration at 2°C; (2) filtration at 25°C, and stored for 3 h; (3) boiled for 30 min at 102°C, and then analysed. The antioxidant properties were evaluated in vitro as antioxidant activity (AA) (model system β-carotene-linoleic acid) and ex vivo as protective activity (PA) against rat liver cell microsome lipid peroxidation measured as 2-thiobarbituric acid-reactive substances (TBA-RS) generated by peroxide degradation. All the vegetable juices showed high but very variable AA ($>83\%$) and PA ($>64\%$). After dialysis and analysis of fractions it was shown that the vegetable contained both biological antioxidant and prooxidant compounds. The prooxidants had MW <3000, conversely the very active antioxidants (PA $=100\%$) had MW $>15\,000$. Electrophoretic analysis revealed that the most active fraction was a complex mixture of brown components at MW $>300\,000$.

Keywords: Antioxidants; Cichoriaceae; Lipid peroxidation; Liver microsomes; Prooxidants

1. Introduction

Reactive oxygen substances (ROS) and reactive nitrogen substances (RNS) involved in a number of chronic diseases (cardiovascular [1,2], neoplastic [3,4], inflammatory neurodegenerative pathologies [5]) and in ageing are continuously generated in the aerobic organism.

Epidemiological studies have pointed out clear relations between diet and such diseases and the protective effects following the consumption of vegetables and fruit [6–8].

For a long time antioxidant vitamins and β-carotene were considered responsible for the positive effects. In recent years it became apparent that other dietary vegetable components, in particular phenols which are ubiquitous in plants, probably contribute to the antiradical and antioxidant effects [9,10].

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A great number of research projects are being carried out to evaluate antioxidant activity (AA) of edible vegetables and medicinal plants [11–13], their extracts [14] and some natural compounds isolated from them [15,16], both in vitro and in the biological systems.

In the present research we set out to investigate the water soluble pro-antioxidant and antiradical in vitro [17–19] and ex vivo [20,21] activities of *Cichorium intybus* juice and to study the features of the active components, against oxidative degradation of lipids. These activities were determined on vegetable juice obtained by simple centrifugation and filtration, to minimize the effect of handling.

The in vitro AA was determined by the widely used model system containing β-carotene-linoleic acid [22]. The ex vivo AA was evaluated as protective activity (PA) against lipid peroxidation of microsome membrane hepatocytes induced by CCl_4 [23]. This xenobiotic induces rapid extensive pathological changes in liver tissue that are well defined at the biochemical and ultrastructural levels [24].

The isolation of the unknown active components was obtained by dialysis and electrophoretic methods.

2. Experimental

2.1. Sample preparation

The vegetable (*C. intybus* var. *Silvestre*, production zone: Chioggia, Italy) was purchased in the winter from a local supermarket. It was washed, weighed, cut into small pieces, homogenized and then centrifuged for 4 min to completely separate the juice from the solid portion. A total of 61.02 ml of juice were obtained from 100 g of fresh vegetable (R.S.D. = 6.771). The juice was filtered on Ruudfilter Schleicher Schuell 1573 (no. 314709, diameter 190 mm) and then on Millipore membranes of cellulose acetate/cellulose nitrate mixed esters (0.45 μm) and was subdivided into three lots. Two lots were filtered in an ice bath (2°C) while the last batch was filtered at room temperature (25°C). The first batch filtered at 2°C was immediately analysed and the second batch was boiled at 102°C for 30 min. All these operations were carried out in dim light. The batch filtered at 25°C was maintained at the same temperature in normal light for 3 h before analysis.

2.2. Dialysis

Dialysis was performed in Spectra/Por Biotech cellulose ester membrane with molecular weight cut-offs 500, 3133, 15 000, 25 000, 50 000, 100 000 and 300 000. A 10 ml aliquot was fractionated by dialysis in 1000 ml of bidistilled water for 24 h at 4°C. The standard substances used to test the dialysis membranes were recovered at a percentage which was higher than 80%. The retentate and the dialysate were brought to the corresponding volumes of juice and tested for PA or freeze-dried and the residues were dissolved in 0.5 ml of bidistilled water for electrophoretic analysis.

2.3. Isoelectric focusing

The samples prepared as described were analysed with isoelectric focusing (IEF) using Pharmacia Constant Power Supply ECPS 3000/150 and Multiphor II electrophoresis unit connected to MultiTemp II thermostatic circulator. The samples (20 μl) were applied directly on Ampholine PAG plate (pH range 3.5–9.5) and the electrode solutions were H_3PO_4 (1 M) and NaOH (1 M). The focusing was run for 60 min with constant power (30 W), up to current 50 mA and voltage 1500 V.

2.4. Acidic hydrolysis

Vegetable juice, filtered at 2°C, and the retentates (MW > 15 000 and 300 000) were refluxed with the same volume of 2 N hydrochloric acid for 15 min. The reaction mixture was centrifuged for 10 min at 4000 rpm to separate precipitate from supernatant. In order to dissolve the precipitate, the latter was adjusted to pH 9 with NaOH (10% w/v). The supernatant was adjusted to pH 7. The precipitate and the supernatant were brought to the corresponding volumes of juice and were tested for PA.

2.5. In vitro antioxidant assay (AA%)

The AA of the vegetable juice, based on coupled oxidation of β-carotene and linoleic acid, was evaluated following the method of Taga et al. [22] with some modifications introduced by Gazzani et al. [19]. β-carotene (5 mg) (Merck) was dissolved in 50 ml of chloroform solution. A 3 ml aliquot of β-carotene chloroform solution was added to a conical flask along with 40 mg of linoleic acid (Merck) and 400 mg of Tween 20 (Merck). Chloroform was evaporated to dryness under reduced pressure at low temperature (less than 30°C). Distilled water (100 ml) was added to the dried mixture, and the mixture was shaken. Four aliquots (400 μl) of vegetable juice, were added to 5 ml of β-carotene emulsion in test tubes, and the mixture was mixed well (samples). In preliminary tests the adding of juice showed no significant change in sample pH. For one sample, the absorbance at 470 nm was immediately measured using the spectrophotometer, and for the other samples absorbance was measured after 10, 20, and 30 min of incubation in a water bath at 50°C. Each sample was read against an emulsion prepared as described but without β-carotene (blank). To correct for the influence of the juice colour in calculating β-carotene degradation rate (dr), four aliquots (400 μl) of the juice were added to 5 ml of blank (blank samples). These mixtures for each time point were spectrophotometrically read, and the absorbance measured was subtracted from that of the corresponding samples. The dr of β-carotene was calculated by first-order kinetics:

$$(\ln(A_0/A_t))/t = \text{dr of sample}$$

where A_0 = absorbance of the sample-absorbance of blank sample at time 0 (absorbance was read immediately after the addition of juice), A_t = absorbance of the sample-absorbance of blank sample at time t, and $t = 10$ or 20 or 30 min of incubation at 50°C.

$$(\ln(a_0/a_t))/t = \text{dr of control sample}$$

where 400 μl of distilled water was added to 5 ml of β-carotene emulsion and treated as the corresponding sample, a_0 = absorbance of the control sample at time 0, and a_t = absorbance of the control sample at time t.

AA was expressed as the percentage of inhibition relative to the control using the equation:

$$\text{AA\%} = \frac{\text{dr control sample} - \text{dr sample}}{\text{dr control sample}} \cdot 100$$

2.6. Biological lipid peroxidation assay (protective activity, PA%)

Liver microsomes were prepared from male Wistar rats weighing 250–300 g following the method of Horie et al. [23] with some modification introduced by Gazzani et al. [21]. The microsomal pellets obtained were suspended either in 0.1 M sodium phosphate buffer, pH 7.4 (control sample), or in vegetable juice (sample) to make a total volume of 6 ml. An aliquot (0.1 ml) of the obtained suspension was immediately removed and used for determination of the microsomes [25].

The remaining preparation was added to NaCl (1 ml, 140 μM), EDTA (1 ml, 50 μM), and sodium phosphate buffer (1 ml, 0.1 M, pH 7.4) and then subdivided into two aliquots of 4 ml, respectively. All samples were stoppered, and N_2 was bubbled through the solution at 37°C for 15 min to obtain anaerobic conditions for the following induction of the lipid peroxidation.

To one group of samples were then added NADP (0.5 ml, 500 μM), G6P (0.5 ml, 250 μM), and CCl_4/EtOH (20 μl, 50% v/v). An equivalent amount of buffer was instead added to the second group.

Both samples were placed in a shaking water bath at 37°C for 30 min, and then the equal volumes of 30% trichloroacetic acid (TCA) at 0°C and 0.75% thiobarbituric acid (TBA) [26] were added.

The reaction mixtures were heated in boiled water for 10 min at 3133 rpm to separate corpuscolate particles.

The absorbance of supernatant was read in a spectrophotometer ($\lambda = 545$ nm) using the second series of samples to bring the spectrophotometer to zero to correct for interference due to colour

and thiobarbituric acid-reactive substances (TBA-RS) naturally occurring in vegetable juices.

The PA was expressed as the percentage decrease of TBA-RS relative to the control using the equation

$$PA\% = (a - b)/a \times 100$$

where a is the TBA-RS in control sample and b represents the TBA-RS in sample.

3. Results and discussion

Table 1 reports the AA and PA values of the clear brown juices obtained by simple centrifugation from *C. intybus* filtered at 2°C. The table also reports the results obtained from juices after storage at 25°C for 3 h, and boiling at 102°C for 30 min. AA increased during reaction time so that at the end of the monitoring period the rate of β-carotene degradation was decreased by more than 80% with respect to unboiled juices and by 94% with respect to boiled juice. It should be pointed out that the variability in AA values decreased in the case of all juices during the reaction time and was lower for the boiled juice. The value of R.S.D., the higher initial (10 min) AA values of boiled juice seem to indicate that anti and prooxidant compounds are present together in the system *C. intybus*. The AA value probably depends on the ratio between their concentrations. The prooxidant compounds, some of which could be enzymes, are not stable on heating so that at the end of the monitoring period in the case of fresh juices, and during the whole reaction period in the case of boiled juice, the AA is mainly due to the type and concentration of the antioxidant compounds.

There is no significant difference between the AA of the juices obtained at 2 and at 25°C at the end of the monitoring period, while significative differences were found among these raw juices and the boiled juice which showed higher AA.

Considering PA, the juice filtered at 2°C, was always found to have PA, but the obtained values were very variable. Stored juice at 25°C showed higher PA which was less variable, and boiled juice was less protective with about the same variability.

To achieve preliminary information regarding the juice's ex vivo protective components, the raw juice filtered at 2°C which has a pH 6.4, was treated with either a base or an acid. Alcalinization made the juice darker, while acidification, from pH 5.3, caused the formation of a dark-brown precipitate which was shown to be completely protective (PA = 100%). The orange–brown coloured supernatant was weakly prooxidant (PA = −11%) (Fig. 1). This indicates that at least some of the active compounds have an acidic character and, in fact, they are insoluble in acidic media.

The juice was then submitted to acidic hydrolysis which produced a dark brown precipitate showing completely PA (PA = 100%), while the supernatant became antioxidant (PA = 64%). This probably could be explained by the fact that the hydrolysis generates acidic media soluble compounds with PA.

Table 1
In vitro antioxidant activity (AA%) and ex vivo protective activity (PA%) of *Cichorium intybus* juice filtered at 2°C, filtered and stored at 25°C for 3 h, and boiled at 102°C for 30 min[a]

Sample	AA%	R.S.D.	AA%	R.S.D.	PA%	R.S.D.
	10 min		30 min			
2°C	30	40.8	83	14.9	64	19.4
25°C	42	37.4	84	13.6	88	14.3
102°C	67	20.8	94	5.3	65	15.6
BHT 0.5 mg ml^{-1}[b]	81	1.2	90	0.5	57	3.7

[a] Values represent means of ten replications.
[b] Gazzani et al. [19,21].

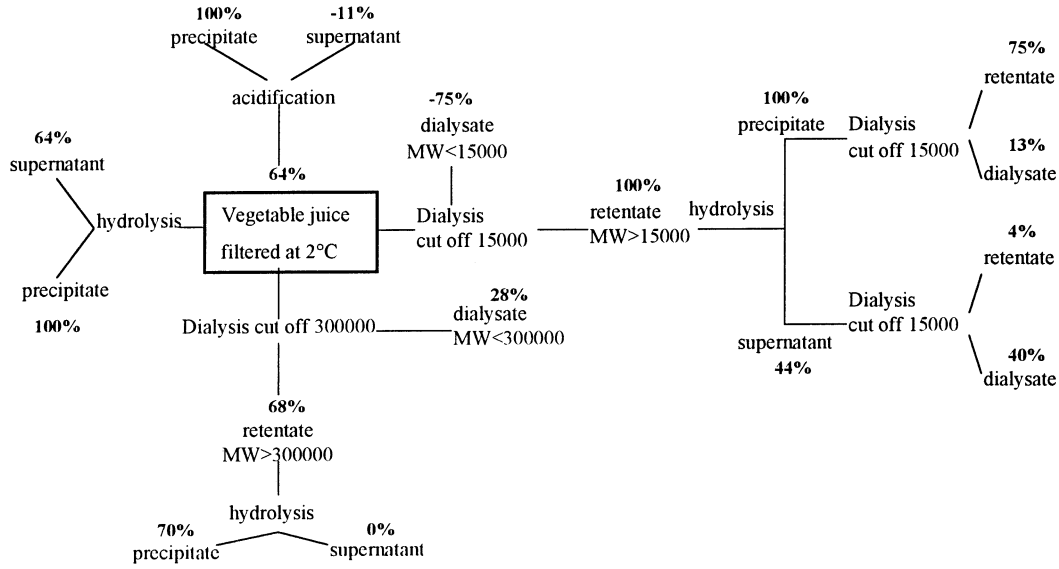

Fig. 1. Protective activity (PA) values (%) of the fractions obtained after acidification and hydrolysis.

The raw juice components were submitted to a preliminary separation by preparative gel filtration chromatography (GFC TSK 40 resin with exclusion limits: 100–10 133, Toyopearl; mobile phase: bidistilled water; flow rate: 0.5 ml min^{-1}, UV detection at $\lambda = 254$ nm) to yield four fractions. Only the higher MW fraction showed PA (PA = 100%). The analysis was repeated using a stationary phase with higher exclusion limits (exclusion limits 1000–100 000). Among the five fractions obtained, the first showed PA (PA = 48%), while fractions 4 and 5 were prooxidant (PA = −22 and −18%, respectively), and the others showed no appreciable activity.

The juice was then dialysed using membranes with increasing cut off values (500–300 000). The sequential dialysis fractions with their PA values are shown in Fig. 2 and Table 2 reports the pH values and dried residues of all fractions obtained. The retentates with MW > 3000, 15 000 and 25 000 all showed PA = 100%, while the dialysate with MW < 3000 was strongly prooxidant. This confirms that the activity of the whole juice depends on both antioxidant and prooxidant compounds present in the system. When freed of prooxidant components the system became completely protective. Using dialysis membranes with higher cut-offs the PA of retentates decreased while the PA of dialysates showed weak or no activity.

The prooxidant fraction (MW < 3000) was dialysed using a membrane with a cut off value of 500 and yielded both a prooxidant dialysate and retentate indicating the presence of more then one prooxidant compound.

The retentate with MW > 15 000 was hydrolysed (Fig. 1). The precipitate and the supernatant obtained showed PA = 100 and 44%, respectively. This confirms that on hydrolysis compounds with PA were generated. In order to establish the MW of active compounds obtained on hydrolysis, both the supernatant and the precipitate were dialysed (membrane cut-off 15 000). Almost all the AA of the supernatant was found in the dialysate. This shows that the generated compounds had low MW. Conversely the activity of the insoluble components in acidic media remained mainly in the fraction with MW > 15 000 (PA = 75%).

The retentate with MW > 300 000 obtained directly from juice was also hydrolysed giving a supernatant and a precipitate with PA = 0 and 70%, respectively (Fig. 1). This seems to indicate that the antioxidants that are generated during

the hydrolysis process are derived from the lower MW active components.

All the fractions obtained with sequential dialysis were submitted to electrophoretic analysis. They all show very complex compositions of the retentates.

4. Conclusions

The results obtained thus far, indicate that *C. intybus* contains both prooxidant and antioxidant compounds which can act in either chemical or biological systems.

The colourless prooxidant compounds active in the biological systems tested are of low molecular weight (< 3133) and are soluble in acidic media. The antioxidant compounds not acid soluble all present MW > 25 000. The most active brown fraction is not soluble in acidic media and has MW > 300 000. The electrophoretic analysis shows it is a complex mixture of brown components. The simultaneous presence of all the antioxidants at the same concentration occurring in

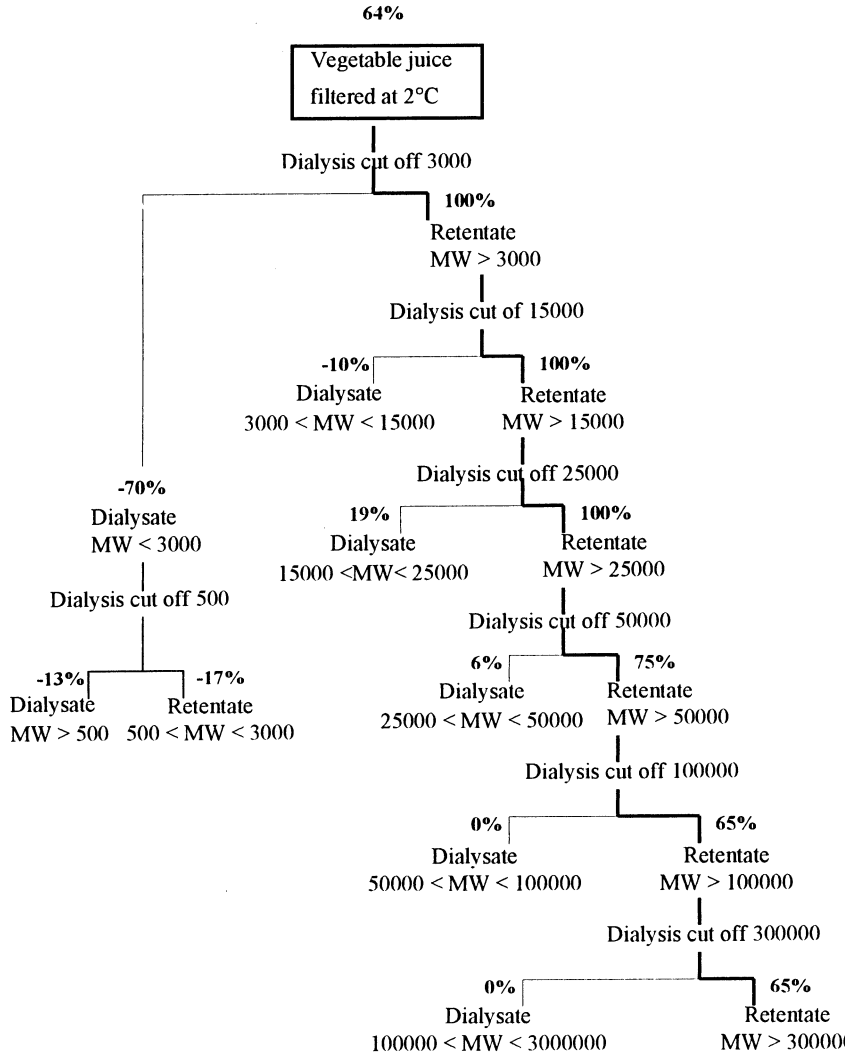

Fig. 2. Protective activity (PA) values (%) of the different fractions obtained by sequential dialysis of raw juice.

Table 2
pH values and dried residues of *Chicorium intybus* juice filtered at 2°C and of fractions obtained by sequential dialysis

Sample	pH	mg ml^{-1}
Vegetable juice 2°C	6.27	41.70
MW < 3133	6.26	28.50
MW > 3000	5.96	12.80
3000 < MW < 15 000	7.55	3.00
MW > 15 000	6.30	9.30
15 000 < MW > 25 000	7.61	4.80
MW > 25 000	5.91	3.80
25 000 < MW < 50 000	7.78	0.75
MW > 50 000	5.80	3.20
50 000 < MW < 100 000	8.00	0.50
MW > 100 000	5.76	2.70
100 000 < MW < 300 000	8.33	0.50
MW > 300 000	5.56	2.30

C. intybus is required to completely inhibit peroxidation of microsomal system lipids. The lower activity of the whole juice with respect to the mixture of all the antioxidants (MW > 25 000) without the prooxidant components, shows that the latter are only able to weaken the PA of the antioxidants.

Investigations are currently under way to better isolate the highly active compounds and to characterize their chemical structure.

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