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Anti- and pro-oxidant activity of water soluble compounds in *Cichorium intybus* var. *silvestre* (Treviso red chicory)☆

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Abstract

The anti- and pro-oxidant activity of water soluble components in *Cichorium intybus* var. *silvestre* was investigated. This vegetable is domestically known as Treviso red chicory recalling its cultivation in the area of Treviso (Italy). The vegetable juices, obtained by centrifugation of the vegetable and treated at 2 and 102 °C, were assessed for their antioxidant activity (AA) using the micellar model system linoleic acid–β-carotene. The obtained juice at 2 °C possessed either anti- or pro-oxidant activity. The boiled juice showed only strong AA, proving that the vegetable pro-oxidant components were thermally instable. Juice components were fractionated by sequential dialysis (1000–300 000 Da membrane cut-off), SPE, GFC, and RP-HPLC techniques showing the presence of several highly antioxidant components with different molecular weight (MW), and polar features in Treviso red chicory. The pro-oxidant fraction capable of masking the presence of the antioxidant components in the vegetable juice is retained by a MW > 300 000 Da dialysis membrane.

Keywords: Diet vegetable; Chicoriaceae; Antioxidant activity; Pro-oxidant activity; Peroxyl radical

1. Introduction

Lipid peroxidation, an auto-catalytic chain reaction, determines negative modifications in taste, flavour and the nutritive value of fats, oil, and fat rich products. Antioxidant compounds are often added to drugs, cosmetics and foods to prevent lipid component degradation. Since synthetic antioxidants could possible have a role in promoting carcinogenesis and being generally considered safer for human health, over the last decade plant materials have been intensively investigated as potential sources of natural antioxidants. On the other hand, lipid peroxidation causes deep alterations in the permeability and functionality of cell membranes in living organisms. Furthermore, free radicals coming from hydroperoxyde decomposition may contribute to a number of chronic diseases such as cardiovas-

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cular, neoplastic, inflammatory or neurodegenerative pathologies, cataracts, diabetes and aging [1–3].

Epidemiological studies have pointed out the protective effects deriving from the consumption of vegetables and fruit against the cited chronic pathologies, and have further increased research on the antioxidant properties of edible vegetables and medicinal plants [4–7].

In our previous reports we have studied many of the most common vegetables within the Mediterranean diet [8,9].

The aim of this research was to evaluate the antioxidant activity (AA) of the water soluble components of the *Cichorium intybus* var. *silvestre*, grown in the Treviso area (a NE city of Italy), commonly called Treviso red chicory IGP (protected geographical indication), and easily considered a traditional Italian food.

The AA% was determined using the micellar system linoleic acid–β-carotene in which the peroxyl radical, induced by heating, attacks β-carotene to give its leucoderivate.

2. Materials and methods

2.1. Sample preparation

Treviso red chicory (*C. intybus* var. silvestre) was purchased during the winter season from a local supermarket.

The vegetable was washed, weighed, cut into small pieces, homogenised, and then centrifuged for 4 min to completely separate the juice from the vegetable material. 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) in an ice bath (2 °C). Filtration resulted in loss of most of the juice colouration. The filtered juice at 2 °C was subdivided into two batches: the first batch was analysed immediately while the second one was boiled for 30 min, (the time commonly used in home cooking of most vegetables). The temperature during boiling was experimentally measured and was found to be 102 ± 0.5 °C. The volumes, the pH values and dry residues (Table 1) of raw and boiled juices were measured.

2.2. 'In vitro' antioxidant assay

The AA of the vegetable juices, based on the coupled oxidation of linoleic acid and β-carotene, were evaluated following, with some modifications, the method of Taga et al. [10]. Some 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 until dry under reduced pressure, and at a low temperature (less than 30 °C). Distilled water (100 ml) was added to the dried mixture, and the mixture was shaken. Five aliquots (400 μl) of vegetable juices were added to 5 ml of β-carotene emulsion in test tubes, and the mixture was mixed thoroughly (samples). In preliminary tests the addition of juices showed no significant change for the 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

Table 1
Description of tested vegetable

Common name	Latin name	ml g^{-1a}	R.S.D.	Thermal treatment (°C)	pH	mg ml^{-1b}
Chicory of Treviso	*C. intybus* var. *silvestre*	0.57	0.05	2	6.14	49.11
	Cultivar of Treviso			102	5.85	39.01

Values represent mean of eight replications.
[a] A ml of raw juice obtained from 1 g of the tested vegetable.

min of incubation in a water bath at 50 °C. Each sample was read against an emulsion prepared as described above but without β-carotene (blank). To correct for any influence from juice colouring during the calculation of β-carotene degradation rate (dr), four aliquots (400 μl) of each juice were added to 5 ml of blank (blank samples). These mixtures were spectrophotometrically read out for each time point, and the absorbance measured was subtracted from that of the corresponding sample. The dr of β-carotene was calculated using first-order kinetics:

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

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

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

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 sample at time t.

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

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

A 100 μM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C®) (Aldrich) solution was also assayed for AA.

2.3. Dialysis

Dialysis was performed in Spectra/Por Biotech cellulose ester membrane with different molecular weight (MW) cut-offs (1000–3500–25 000–300 000 Da). A 4 ml aliquot of Treviso red chicory filtered at 2 °C was fractionated by dialysis in 400 ml of Millipore grade distilled water for 24 h at 4 °C. The standards used to test the dialysis membrane were recovered at a percentage higher than 80%. The retentate and the dialysate were brought up to the corresponding volume of juice and tested for antioxidant assay.

2.4. Solid-phase separation of the vegetable juice (SPE)

The MW < 1000 Da fraction obtained by Treviso red chicory juice filtered at 2 °C was separated into two fractions using a Bakerbond C_{18} SPE cartridge. The C_{18} cartridge was preconditioned with 4 ml of Millipore grade distilled water, and then with 4 ml of methanol. After the sample was loaded (0.5 ml), the C_{18} cartridge was washed with 10 ml of water and C_{18} bound compounds were eluted with 10 ml of methanol. After evaporation of the solvents, each fraction (bound and unbound) was dissolved in a 0.5 ml aliquot of bi-distilled water and tested for antioxidant assay.

2.5. Gel filtration chromatography (GFC)

The bound and unbound fractions were separated into sub-fractions by preparative gel filtration chromatography (GFC) using a Merck superformance universal glass cartridge system 300 × 10 mm i.d. column, first with Toyopearl HW 40(F) packing (molecular weight separation range: 100–10 000). The system was equipped with a Waters 490E UV–Vis detector and a Hitachi-Merck D2500 integrator; the mobile phase was distilled water and the flow rate was 0.8 ml min^{-1}; UV detection was at 270 nm. After evaporation of the solvent, each fraction was brought to the original juice volume and tested for antioxidant assay.

Blue dextran (MW = 2 000 000, retention time = 10.8 min), 3′,3″-bis-[*N*-(carboxymethyl)amino-methyl]thymolsulfonphthalein) (glycine thymol blue MW = 662.7, retention time = 14.37 min), vitamin B12 (MW = 382, retention time = 21.72 min), caffeine (MW = 194, retention time = 36.6 min) were used as standards.

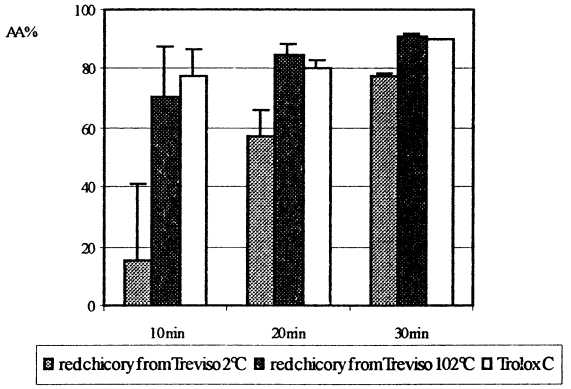

Fig. 1. Antioxidant activity (AA%) of raw and boiled Treviso red chicory juices and of 100 μM Trolox C® solution.

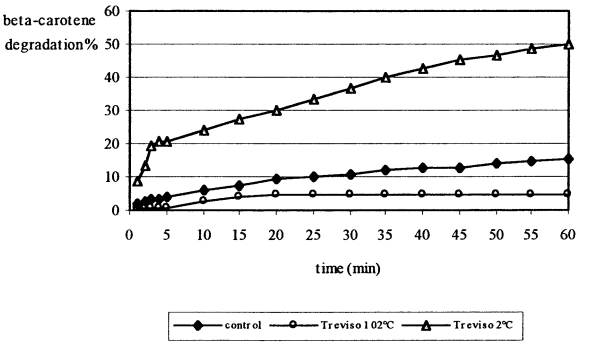

Fig. 2. β-Carotene degradation percentage in absence, and in presence, of raw and boiled Treviso red chicory juices.

3. Results and discussion

The Treviso red chicory water soluble components were analysed for their AA%. They were separated as a solution from the whole homogenised vegetable by simple centrifugation to minimise the effects of handling. The volume of raw juice obtained from 1 g of vegetable, the pH, the dry residue left over by 1 ml of raw and at 102 °C treated juices was reported in Table 1. The antioxidant activities were determined for the raw and boiled juices, on the basis of the inhibition percentage of the β-carotene degradation rate and expressed as AA%, determined after 10, 20, 30 min of reaction at 50 °C using the model system linoleic acid–β-carotene in presence of, and without, the vegetable juices. The results obtained are shown by the histograms of Fig. 1. The chicory initially (after 10 min of reaction) showed, on the average, a rather weak AA% (due to the initially pro-oxidant activity of some vegetable components) which then increased with time (AA_{20}) reaching a value close to 80% at the end of the monitoring period (AA_{30}). At this point, the AA value of a 100 μM Trolox C® solution, used as antioxidant standard, was 93%. The boiled juice, separating a precipitate during boiling, right from the beginning, showed high antioxidant activity (AA = 72%), which at the end of the reaction time was higher than that of the raw juice (AA = 89%) and very close to that of the AA% of the Trolox C® solution.

The components precipitated during the boiling period, separated and tested for their activity, at least after boiling, showed no pro-oxidant activity.

Table 2
AA% of juice fractions obtained by dialysis

Fraction	Sample	AA%			mg ml^{-1}[a]
		10 min	20 min	30 min	
	Raw juice	−62	28	62	56.32
A	MW < 3500 Da	62	78	90	47.90
B	MW > 3500 Da	−66	21	53	7.35
C	3500 Da < MW < 25 000 Da	68	81	90	0.83
D	MW > 25 000 Da	−49	52	56	6.12
E	25 000 Da < MW < 300 000 Da	71	78	84	1.95
F	MW > 300 000 Da	−34	24	50	4.06

Values represent mean of three replications.
[a] Dry residue per ml of tested vegetable.

Considering the different profile for the increase of the AA values observed during the monitoring time for the raw and the thermally treated juices, both of these were tested again using the model system linoleic acid–β-carotene, without heating, to accelerate the peroxidation of the linoleic acid, and measuring the degradation of β-carotene every minute for the first 5 min, and followingly every 5 min for 1 h. The degradation rate was much faster in presence of the raw juice compared with the control sample, even after only a minute of reaction, indicating no induction period in the

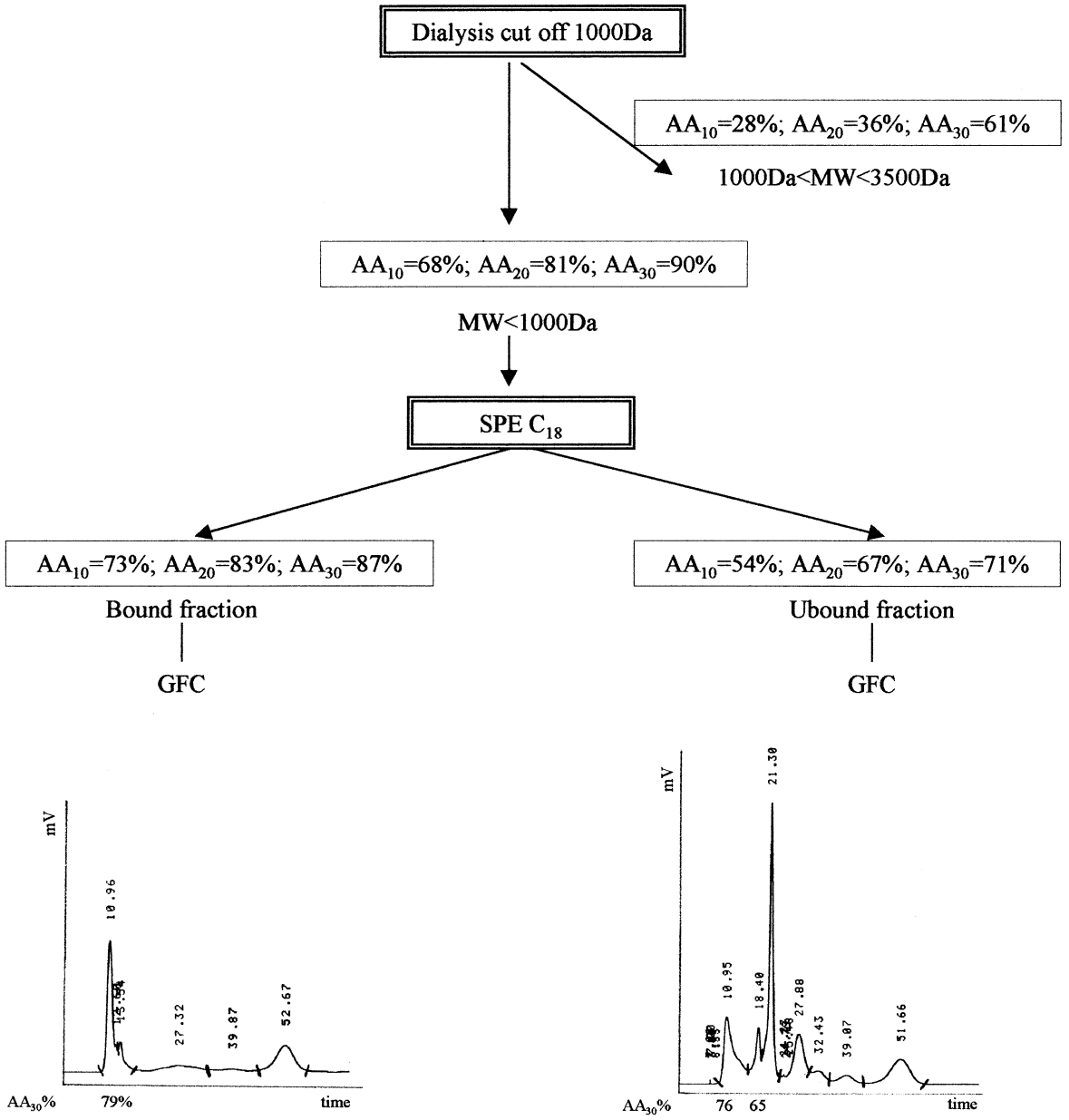

Fig. 3. Isolation scheme of antioxidant raw Treviso red chicory juice components.

acid linoleic peroxidation just as a lipoxygenase enzyme acts (Fig. 2). The pro-oxidant activity lasted throughout the whole monitoring period (60 min). Conversely, the boiled juice showed itself to be promptly antioxidant with persisting and increasing activity indicating the presence of thermally stable antioxidant and instable pro-oxidant components in the vegetable under investigation.

To obtain preliminary information about the components responsible for the anti and pro-oxidant activity of the raw juice, its components were fractionated on the basis of their MW by dialysis, sequentially using membranes with different cut-offs (3500–25 000–300 000 Da). The AA% of the obtained fractions is reported in Table 2. Even the A, C and E fractions initially showed positive AA% values always higher than that of the raw juice. Conversely, the B, D and F fractions were all initially pro-oxidant and then became antioxidant with values lower than those of the raw juice at the end of the reaction period. The antioxidant fraction AA values always being higher than those of the raw juice suggests that the pro-oxidant components are able to contrast or mask the action of the antioxidant compounds. These compounds could immediately be revealed only after separation or thermal inactivation of the pro-oxidant components, as shown by the AA values of the fractions obtained by the dialysis, with 3500 Da membrane, of the boiled juices. Sequential dialysis separation of the initially pro-oxidant fraction with MW > 3500 Da (B), carried out first with cut-off 25 000 Da and then with cut-off 300 000 Da membranes (C and E fractions), showed that the pro-oxidant activity must be attributed to the fraction retained by a 300 000 Da dialysis membrane. Conversely, sequential dialysis showed in the Treviso red chicory at least three components with very strong anti-oxidant activity (A, C and E fractions), promptly evident when separated from pro-oxidant components.

The study was continued on the antioxidant components (Fig. 3). Therefore, MW < 3500 Da antioxidant fraction was dialysed again (cut-off 1000 Da membrane) and the smallest MW and most active components were considered. Preliminary GFC analysis showed an overly complex mixture, therefore, its components were further separated on the basis of their polarity by a Baker C18 cartridge. The GFC analysis of both the obtained fractions showed they remain complex mixtures. The bound (less polar) fraction was divided into four sub-fractions, the first of which showed antioxidant properties. The unbound (more polar) fraction gave six sub-fractions, the two less retained of which resulted as active (Fig. 3). These three antioxidant fractions all had retention times lower than that of 3′,3″-bis-[*N*-(carboxymethyl)amino-methyl]thymolsulfonphtalein) (glycine thymol blue) used as a standard (MW = 662.7).

4. Conclusion

The obtained results showed that Treviso red chicory contained several water soluble components able to strongly contrast lipid per-oxidation. Their activity could be entirely observed only after the inactivation or elimination of pro-oxidant components which also occurs in the vegetable juice. The AA value given by the whole juice was the result of several antioxidant compounds' many different actions, and a fraction with pro-oxidant properties. The antioxidant compounds had different polar characteristics, and MW was higher than 700 Da for all the antioxidant fractions found. The pro-oxidant fraction, which was retained by a 300 000 Da membrane, strongly accelerated the lipid per-oxidation rate and its activity persisted for a long time at 25 °C. It was able to annihilate or completely mask the action of the antioxidant compounds, but its activity completely disappeared in 20 min at 50 °C, or after boiling the vegetable juice. These features and the fact that pro-oxidant activity was not observed in the deoxyribose assay, [11] which uses a system not containing linoleic acid (data not shown), seem to indicate that a lipoxygenase, enzyme widely occurring in plants, may be responsible for the pro-oxidant activity registered.

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