Naturally occurring or induced by thermal treatment food components promoting intestinal health

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The research carried out has concerned the antioxidant and antiradical activities of water soluble components occurring in red chicory (Cichorium intybus var. Silvestre), champignon mushroom (Agaricus bisporus) and barley coffee (Hordeum vulgare). In order to evaluate these activities chemical (DPPH assay and linoleic acid-β carotene system), enzymatic (xanthine-xanthine oxidase system and superoxide radical anion scavenger activity), and ex-vivo biological assay (liver microsome system) have been used. The active components were isolated using dialysis procedure and GFC technique. The best antioxidant activity was found in mushroom juice which contains high and low MM components, very active both in chemical and biological assays. The active fractions in all food matrix have shown to be affected by the gastric conditions, that cause a partial hydrolysis. Nevertheless, this seems to have a positive effect. In fact, the mixture obtained after hydrolysis shows towards the microsomal system a protective activity even higher than the vegetable juice.

1 Introduction

Over the last twenty years, an always stronger relationship has emerged between food components and health. As a matter of fact, the diet can influence both positively and negatively our health state. Positive effects are attributed to the presence in foods of such components, not traditionally considered, that contribute significantly to the overall well-being and health of our organism through different actions and mechanisms.

Among such food components, a great interest has concerned polyphenolic derivatives which, due to their antioxidant properties, are able to contrast radicals activity slowing down the aging process and preventing or putting off the onset and progression of several serious chronic pathologies with a strong social and economical impact, such as cardiovascular, inflammatory, neurodegenerative diseases, certain types of cancer, diabetes, cataract. Furthermore, antioxidant compounds in foods are particularly active against gastro-intestinal cancer, due to their in situ ability to reduce oxidative stress.

Several studies have been carried out on food components known as prebiotics. These substances are non-digestible in the upper intestinal tract and therefore can not be considered nutrients, but can be fermented in the colon by the intestinal eubiotic flora. In particular, a great interest has been shown in non-digestible oligosaccharides, such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) [1]. Such molecules modify the intestinal micro-flora promoting bifidobacteria and lactobacilli growth and lowering pathogens growth, often responsible of toxins production, and induce positive systemic effects for human health [2,3].

It has also been shown that a high calories diet, rich in fat content and poor in fibers, is associated with an increase in intestinal tumors; on the other hand, fiber rich diets, meaning a high consumption of fruits and vegetables, seem to play a protective role towards these pathologies. Within the factors correlated to such pathologies, of paramount importance are age and inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn’s disease, a chronic inflammation of characteristic tracts of the intestines. Therefore, the most accredited hypothesis regarding the etiopathogenesis of IBD claims that, in genetically susceptible subjects, an altered function of the intestinal mucosa can be observed, leading to a chronic activation of immune and inflammatory processes by intestinal microflora endoluminal antigens. The
“non-controlled” inflammation causes the immune-inflammatory process to become chronic. It also has to be noticed that the intestinal mucous membrane damage, that occurs with these diseases, can be caused by an excessive production of highly reactive oxidant radical species (such as superoxide anion, hydrogen peroxide, ipochloric acid and nitrogen monooxide) compared to the activity of the antioxidant defence mechanisms of the unhealthy organism [4].

Moreover, it was demonstrate that the inflammatory intestinal disease was associated with an higher xanthine-oxidase activity. Therefore, the oxidative stress and the altered microbic flora have been identified as key elements responsible for tissue damage accompanying the inflammatory bowel diseases. The intake of foods containing substances with antioxidant or prebiotic activity, able of crossing the gastric barrier in their active forms, could represent an interesting approach to reduce the intestinal damage and consequently the onset of IBD, which in turn are risk factors for colon cancer [5-7].

2 Material and Methods

2.1 Chemicals

Phosphate buffer (pH 7.4 and pH 3), borate buffer (pH 10), 2,2’-diphenyl-1-picrylhydrazil radical (DPPH), linoleic acid, β-carotene, Tween 20, ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G6P), trichloroacetic acid (TCA), xanthine, xanthine oxidase (XO), nitro blue tetrazolium chloride (NBT), superoxide dismutase (SOD) and allopurinolo were purchased from Sigma-Aldrich S.r.l. Sodium chloride, sodium phosphate buffer, carbon tetrachloride (CCl₄), thiobarbituric acid (TBA), o-phtalaldehyde (OPA), 9-fluorenylmethyl chlororformate (FMOC), butylhidroxiltoluol (BHT) and methanol (spectrophotometric grade > 99%) were purchased from Carlo Erba.

2.2 Vegetable samples and juice preparation

Red chioggia Chicory and champignon mushrooms were purchased from a local supermarket. They were washed, cut in small pieces, homogenizated, and than centrifugated at 5000rpm for 4 min to completely separate the juices from the vegetables. The juices were filtered through a paper filter and then trough Millipore membranes of cellulose acetate/cellulose nitrate mixed esters (0,45 µm), and the pH value of each sample was measured.

2.3 Roasting barley conditions and Barley solution preparation [8]

Natural barley grains (var. Clarine, courtesy of Crastan-Pontedera) were roasted in a pilot roaster apparatus. They were heated to 70-80°C in 3 min, to 200°C in 30 min and finally to 220°C in 13 min; they were then cooled by water spray to 180-190°C and finally to room temperature with air.

One sample of roasted barley was ground in a laboratory scale mill and sieved through a no. 30 sieve. Barley solution was prepared according to the common Italian procedure for brewed coffee. A 6 g sample of roasted barley was boiled for 10 min in 100 ml of Millipore grade water. The solution was then filtered on Millipore membrane of cellulose acetate/cellulose nitrate mixed esters (0,45 µm), and the pH value of each sample was measured immediately.

2.4 DPPH assay (Antiradical Activity, ARA%) [9, 10]

Antiradicals activity was determined using DPPH as a free radical. A 100 µl aliquot of vegetable juices or barley solution (sample) or a 100 µl aliquot of KH₂PO₄/NaOH buffer (pH 7.4) (control sample) was added to 3,9ml of 6 x 10⁻⁵ mol/L methanol/KH₂PO₄/NaOH buffer (50:50 v/v) DPPH Solution. The decrease in absorbance was determined at 515nm when the reaction reached a steady state (after 20 min of reaction).
The percent scavenger activity (ARA%) against DPPH was calculated in accordance with the following equation:

\[
\text{ARA}\% = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}\right) \times 100
\]

The scavenger activity was also determined for a methanolic solution of BHT (Sigma) which was assayed at the concentration of 0.1mM.

### 2.5 Linoleic Acid β-carotene Assay (Anti-peroxyl radical activity AA%)

The antioxidant activities of vegetable juices and barley solution, based on coupled oxidation of linoleic acid and β-carotene, were determined in accordance with a modified version [10, 11] of the method of Taga et al. [12]. Briefly, 5mg of β-carotene 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 and 400 mg of Tween 20. The chloroform was evaporated until dry under reduced pressure at low temperature (<30°C). Millipore grade water (100ml) was added to the dried mixture, and the mixture was shaken. Two aliquots (100 µg) of juices and barley solution were added to 5ml of β-carotene emulsion in test tubes, and the mixture was thoroughly mixed (samples). One of the sample’s absorbance was immediately measured with the spectrophotometer set at 470 nm, and the other sample’s absorbance was measured after 20 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 any influence that might be due to vegetables or barley solution color in the calculation of the β-carotene degradation rate (dr), an aliquot of each vegetable juices or barley solution was added to 5mL of blank (blank sample). These mixtures were read spectrophotometrically, and the absorbance measured was subtracted from that of the corresponding sample. The dr of β-carotene was calculated by first-order kinetics

\[
\ln\left(\frac{A_0}{A_t}\right)/t = dr \text{ of sample}
\]

where \(A_0\) = absorbance of the sample - absorbance of blank sample at time 0 (absorbance was read immediately after the addition of vegetable juices or barley solution), \(A_t\) = absorbance of the sample - absorbance of blank sample at time t, \(t = 20\) min of incubation in a water bath at 50 °C.

\[
\ln\left(\frac{a_0}{a_t}\right)/t = dr \text{ of control sample}
\]

where 100 µl of Millipore grade water was added to 5 ml of β-carotene emulsion and treated as the corresponding sample, \(a_0\) = absorbance of the sample - absorbance of blank sample at time 0, 
\(a_t\) = absorbance of the sample - absorbance of blank sample at time t.

AA was expressed as the percent of inhibition relative to the control in accordance with the following equation:

\[
\text{AA}\% = \left(\frac{\text{dr control sample} - \text{dr sample}}{\text{dr contro sample}}\right) \times 100
\]

A BHT solution was also assayed for AA at the concentrations 0.1mM.

### 2.6 Biological Lipid Peroxidation Assay (Protective Activity, PA %)

Liver microsomes were prepared from male Wistar rats weighing 200-250 g in accordance with a modified version [11, 13] of the method of Horie [14]. The microsomal pellets obtained were suspended either in 0.1M sodium phosphate buffer (pH 7.4) (control sample) or in vegetable juices or barley solution (sample) to make total respective volumes of 6 mL. An aliquot (0,1ml) of the obtained suspension was immediately removed and used for determination of microsomal proteins [15]. The remaining preparation was added to NaCl (1ml, 140 µM), EDTA (1ml, 50 µM), and sodium phosphate buffer (1ml, 0.1M, pH 7.4) and then subdivided into two respective aliquots of 4ml. All test tubes containing samples were stoppered, and N₂ was bubbled through the solutions at 37°C for 15 min to obtain anaerobic conditions for the subsequent induction of lipid peroxidation. To one group of samples we then added NADP (0.5ml, 250 µM) and CCl₄/EtOH (20µl, 50% v/v). In contrast, we added an equivalent amount of buffer to the second group. Both samples were placed in a shaking water bath at 37 °C for 30 min, and equal volumes of 30% TCA...
at 0°C and 0.75% TBA were subsequently added [16]. The reaction mixtures were heated in boiling water for 15 min, kept in ice for 5 min, and then centrifuged for 10 min at 3000 rpm to separate corpuscolate particles.

Absorbance of supernatants was read in a spectrophotometer (λ = 545 nm) using the second series of samples treated as above but without coenzymes to bring the spectrophotometer to zero. This was done to correct any interference deriving from the color of TBA reacting substances (TBA-RS) that naturally occur in sample solutions.

The protective activity (PA%) was expressed as the percentage decrease in TBA-RS relative to the control in accordance with the equation

\[ PA\% = \frac{(a-b)}{a} \times 100 \]

where \( a \) represents the TBA-RS in control sample and \( b \) the TBA-RS in sample.

The protective activity was also determined for an aqueous/methanolic solution of BHT which was assayed at the concentration of 0.1mM.

2.7 Effect on xanthine oxidase (XO) activity [17]

The effect of the vegetable juices or barley solution on XO activity was evaluated by measuring the formation of uric acid from xanthine in a double-beam spectrophotometer at room temperature.

A total of 480 µl of XO (0.592 U/ml) in a phosphate buffer (0.1M) was mixed with 10 µl of the vegetable juices or barley solution (sample) or water (control). The reaction was started by the addition of 500 µl of xanthine (400 µm) in the phosphate buffer, and the absorbance was recorded at 295nm for 2 min.

The inhibitory activity (IA%) was expressed as the percentage of acid uric formation in the sample relative to the control in accordance with the equation:

\[ IA\% = \frac{(Abs \; control-Abs \; sample)}{Abs \; control} \times 100 \]

The IA% was also determined for a solution of allopurinol which was assayed at the concentration of 10mM.

2.8 Superoxide radical anion (O2−) scavenging activity [18]

The scavenger activity of vegetable juices or barley solution was evaluated by monitoring the reduction of nitro blu tetrazolium (NBT) induced by \( O_2^- \) produced by the xanthine oxidase mediated degradation of hypoxanthine.

Different concentration of juices or barley solution were added to the reaction solution containing 100µl of 30mM EDTA (pH 7.4), 10µl of 30mM hypoxanthine in 50mM NaOH, and 200µl of 1.42mM of NBT. After the solution was preincubated at room temperature for 3 min, 100µl of 0.5U/ml xanthine oxidase was added to the mixture and the volume was brought up to 3 ml with 50mM phosphate buffer (pH 7.4). After the solution was incubated at room temperature for 20 min, absorbance was measured at 560nm.

The scavenging activity (SA%) was expressed as the percentage of NBT reduced in the sample relative to the control in accordance with the equation:

\[ SA\% = \frac{(Abs \; control-Abs \; sample)}{Abs \; control} \times 100 \]

The SA% was also determined for a solution of superoxide dismutase which was assayed at the concentration of 10mM.

2.9 Dialysis [8]

Dialysis was performed with Spectra/Por Biotech cellulose ester membrane and molecular weights cutoff at 3500Da. A 10-ml aliquot of juices or barley solution was fractionated by dialysis in 1000 ml of Millipore grade water for 6 h at 4 °C. The dialysates and the retentates were brought to the initial volume of the samples (10ml) using Millipore grade water before to be tested for DPPH assay, peroxyl radical assay, protective activity assay, xanthine xanthine-oxidase assay and superoxide radical anion assay.
2.10 Gel Filtration Chromatography (GFC) [8]

The GFC apparatus were a 655A-11 Merck-Hitachi liquid chromatograph and a 1100 Agilent liquid chromatograph with a wavelength UV monitor at 210 nm. The GFC separation of the dialysis fractions of each sample vegetables juices and roasted barley fraction was performed with a Superformance Universal glass-cartridge system (300 x 10 mm) (Merck). The stationary phases were at first TSK gel Toyopearl HW-75F (exclusion limits 1000-10000 KDa) (TSK75) (Tosoh Biosep GmbH).

The mobile phase was Millipore grade water at a flow of 1 ml/min.

2.11 Hydrolysis [8]

For hydrolytic investigations, 1ml dialysis fraction of the samples was treated with 1ml of 4N HCl, and the mixture was heated for 2 h at 37 °C in a sealed tube. After rapid cooling, the pH of the mixture was adjusted to the initial sample pH, and was then filtered.

2.12 Statistical analysis [8]

The values represent the mean value of, at least 3 replications for the anti-DPPH and the anti-peroxyl radical assay, and 3 independent experiments on duplicate samples for PA, IA and SA assays. Data were analyzed by analysis of variance (ANOVA) with the Statgraphic Plus (1998) statistical package. Means

3 Results and discussion

3.1 CICHORIUM INTYBUS var. SILVESTRE

Chemical and biological assays showed a high antioxidant activity both for the water soluble components occurring in Cichorium intybus var. silvestre (or Chioggia chicory) juice and for its high molecular weight dialysis fraction (retentate MM > 3500 Da). The results obtained for the latter showed that the high molecular weight (HMM) fraction is very active in all the used tests (fig. 1).

In order to isolate and gain more detailed information about the molecular weight of the active compounds, the HMM fraction was separated through GFC (fig. 2) providing five different subfractions analysed with the enzymatic and ex-vivo tests. The obtained results showed that in the biological assay all fractions were active, with the highest values given by the two ones at lower retention times. On the contrary in the enzymatic tests the GFC fractions were not active (fig. 3). The relatively high values found for each fraction indicate that, when together, among the different fractions any interaction develops that inhibits their protective action.

![Fig. 1: ARA%, AA% AP%, AI%, AS% of Chioggia chicory and its dialysis fractions.](image-url)
The effect of pH on the active high molecular weight dialysis fraction was evaluated by incubating it with HCl 2 N at 37°C for 2h. This treatment causes the formation of a precipitate that was submitted to the biological and enzymatic tests together with the supernatant (fig. 4).
Fig. 5: ARA%, AA%, PA%, IA%, SA% of mushroom juice and its dialysis fractions.

The results showed that protective activity is possessed by the hydrolysis products that, altogether, show an even higher activity than that showed by the HMM fraction prior to hydrolysis. Conversely, this treatment causes a decreased capacity of the retentate to inhibit XO activity as well as a weak loss of scavenger ability against the superoxide anion splitted between the supernatant and the precipitate components.

3.2 AGARICUS BISPORUS

The antioxidant-antiradical activity of the Agaricus bisporus (champignon mushroom) in-toto and its dialysis fractions (dialysate MM<3500Da and retentate MM>3500Da) have been evaluated with the chemical, enzymatic and biological assays (fig. 5).

The results of chemical assays show that the juice and the two dialysis fractions possess good and strong antiradical activity in the DPPH and linoleic acid –β-carotene tests, respectively; in the biological assay the juice and both dialysis fractions possess very strong activity, in fact they all are able to inhibit completely the lipid peroxidation in the microsomal system. As regards the radical O₂⁻ anion, good activity is shown by the juice, even higher activity by the HMM fraction and scarce activity by the LMM fraction. These findings show that the active compounds have HMM and that negative interactions between HMM and LMM occur. Conversely all the samples show negligible action in inhibiting the XO activity.

As both the retentate and the dialysate were possess antiradical activity, both were resolved through GFC (exclusion limits 1000-10000 KDa). The retentate was separated in five fractions (fig. 6) all submitted to the enzymatic and ex-vivo tests for antioxidant and inhibitory activity evaluations (fig. 7).

The results of biological assay show that all fractions are active, the first one with the highest molecular weight (FR1), the only one very active also in scavenging O₂⁻, especially.

As regard enzymatic activity inhibition, only FR1 shows weak action against XO.

The dialysate gave five GFC fractions too (Fig. 8). They all were analyzed by biological and enzymatic assays (fig. 9).

As regards the antiradical activity, in the biological assay all fractions showed a good activity, the most active being F4, whereas in the enzymatic assay only FR1 and FR2 showed to be active. All the fractions showed negligible activity against XO enzyme.

The evaluation of the effect of the gastric environment was carried out on both the active dialysis fractions, which were hydrolysed with HCl 2N for 2h at 37°C. Both of them gave a precipitate. The results obtained in the biological system showed that for both the HMM and LMM fractions the protective activity not only was maintained, but even enhanced because supernatant and precipitate given by both the fractions, showed high activity. Hydrolysis caused a loss of scavenging activity in the HMM fraction, whereas increases the activity of LMM fraction in fact the supernatant obtained from dialysate showed higher activity than the non treated dialysate (figure 10). No remarkable changes are shown as regards the negligible anti- XO activity.
3.3 HORDEUM VULGARE

The barley coffee beverage has shown strong antiradical properties in chemical and biological tests and a scarce superoxide anion radical scavenger activity in the enzymatic assay. The enzymatic system also underlined that both the beverage in-toto and dialysis fractions have scarce xanthine-oxidase inhibiting activity (fig. 11).

The research was continued to investigate the components of the HMM fraction that was resolved, by GFC, in three fractions tested by ex-vivo test.

The results showed FR1, corresponding to a pure HMM component, as shown by capillary electrophoresis analysis, completely inhibits microsomial lipid peroxidation, so this component can be considered responsible for most of barley coffee activity. According to the elution time, compared to dextran blue used
Fig. 8: Chromatogram of mushroom MM<3500Da.

Fig. 9: PA%, IA%, SA% of mushroom GFC fractions.

As molecular weight standard, a molecular mass within the range of 1000 and 2000 KDa can be assigned to this macromolecule.

This component was hydrolysed in the acidic condition already described 14).

The hydrolysed mixture showed the same protective activity (PA=100) as the macromolecule before treatment. It is therefore possible to state that, although the acidic environment of the stomach involves a partial hydrolysis of the isolated macromolecule (showed by GFC analysis), the beverage antioxidant and antiradicals properties, useful against oxidative stress conditions that can be established in the gastro-intestinal tract, are maintained.
**Fig. 10:** PA%, IA%, SA% of mushroom dialysis fractions before and after hydrolysis.

**Fig. 11:** ARA%, AA%, PA%, IA%, SA%, of barley solution and its dialysis fractions

**Fig. 12:** Chromatogram of barley solution MM>3500Da.
Fig. 13: PA%, IA%, SA% of barley solution GFC fractions.

The isolated and purified macromolecule has been elementally analysed (CHN), giving a low percentage of N (N% 1.12; C% 39.03; H% 6.92), and the chromatographic analysis with the amino acid analyser showed that it is not amminoacidic nitrogen.

The research has highlighted that barley coffee shows high antioxidant activity in all chemical and biological tests. Responsible for these antioxidant and antiradical activities was found to be a brown coloured macromolecule, having MM ranging between 1000 and 2000 KDa and containing a low percentage of non amino acidic nitrogen. All these characteristics allow us to ascribe this compound to the group of melanoidins.

4 Conclusion

The research had highlighted that Cichorium intybus, var. silvestre, Agaricus bisporus and Hordeum vulgarius, when roasted, contain water soluble compounds possessing powerful antioxidant and antiradical activities in a number of chemical, biological and enzymatic assays. The most responsible for the antiradical activity for all the considered vegetables, resulted to be high molecular weight components. The highest antiradical and antioxidant capacity were found in mushroom juice which contains very active low molecular weight components, too.

The active fractions in the vegetables and in barley coffee have shown to be affected by the gastric conditions that cause a partial hydrolysis. The hydrolysis process in general seems to produce a positive effect because in the whole the antioxidant properties of the obtained mixture are strengthened. On the basis of these findings we can hypothesize that the consumption of red chicory, champignons and barley coffee could be useful to hinder oxidative stress that promotes the onset of bowel chronic diseases.

References


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