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Hydroxytyrosol from Olive Water Added as Antioxidant to Cow Milk

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Abstract

Hydroxytyrosol is the main polyphenols present in the olive water. Among the natural polyphenols, it has strong antioxidant properties, which can find application in human health, as anticancer, anti-inflammatory, for health disease, cholesterol. The results showed that interesting this natural compound could be added to the milk, without lose its antioxidant properties. The largest part of hydroxytyrosol added to the milk could be found in the whey fraction, and only a small part associate to the casein, which is usually known to inhibit the antioxidant properties of polyphenols added to the milk. The results indicated that in the case of polyphenols from olive water, the interaction with casein was reducing and consequently, the antioxidant properties of hydroxytyrosol in the milk were preserved.

The present work shows a new perspective for the utilization of olive water as natural and healthy ingredient for an enriched functional food.

Keywords: Hydroxytyrosol; Polyphenols; Cow milk; Olive water

Introduction

The polyphenols represent a large group of chemical compounds which are ubiquitous (plants, vegetables, fruit, vines, tea, coffee and microalgae). Phenolic compounds are known to be very powerful natural antioxidants, which can act by chelating metal ions, preventing radical formation and improving the antioxidant endogenous system [1].

The importance of resveratrol present in the wine is well known and new positive implication in human health is continuously found [2]. The presence of polyphenols, with triterpenoids and carotenoids, has been identified, conferring antioxidant properties to skin tissue of pear [3].

The addition of polyphenolic substances to foods, not only provides them with an enormous added value from a health standpoint but stabilizes foods without adding more expensive and more harmful preservatives and synthetic stabilizers [4]. For this reason, consumption of polyphenols with the diet can produce an important benefit for human health.

There is an increasing evidence of the benefit for human health derived by consumption of food with great nutritional value and bioactive properties.

Among these, milk and its fermented products are widely considered, so that large kind of different formulations are currently distributed in the food markets. This evidence can attract attention on the utilization of milk enriched with polyphenols for antioxidants intake with the diet.

However, it has been reported that the antioxidant capacity of tea polyphenols could be inactivated by the addition of milk. This can be ascribed to the formation of complexes of catechins (the major polyphenols in the tea) and the milk proteins. Some authors reported how the formation of these complexes would alter their antioxidant activities [5,6]. Other authors on the contrary showed the capacity of casein micelles to preserve antioxidant properties of catechins, by encapsulation of tea polyphenols and acting as vehicle for antiprolferative activity on colon cancer [7]. So the effect of interaction of milk proteins and polyphenols is a controversial subject. However, investigation on the possible application of different source of polyphenols, as additive in milk products, has to be taken into account, for the evidence of the healthiness of polyphenols intake.

In particular, hydroxytyrosol, derived by the hydrolysis of the oleuropein, a polyphenol present in olives, has been shown to be involved in the prevention and treatment of many diseases, such as coronary heart disease and cholesterol, LDL cholesterol, atherosclerosis, inflammation, cancer and many other diseases.

Recent studies showed the bioavailability of hydroxytyrosol recovered from olive mill waste water, which was absorbed and excreted in human urine [8]. These results pointed out that this natural antioxidant compound may represent a promising ingredient of nutraceuticals.

Previous studies showed how it was possible to use polyphenols recovered from olive water, as enrichment of yogurt, both free and encapsulated [9].

Other studies studied the addition of oleuropein as bioactive compound as additive in both milk and yogurt [10], indicating that the addition of this polyphenol was effective in providing a novel functional dairy product. Moreover, other authors analyzed the antioxidant capacity of different fractions of cow milk, comparing the milk, with whey and deproteinized milk, putting in evidence the higher contribution of the whey to the antioxidant capability of milk, with respect to the protein fraction [11].

Although previous studied focused on the utilization of

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polyphenols addition to milk and its fermented products, to present new enriched healthy food, to our knowledge there is no study on the evaluation on the antioxidant and antiradical activity of this products and their stability.

In order to gather more information on the potentiality of mixing milk and polyphenols, we added to commercial milk some polyphenols obtained from concentration of treated olive water.

The aim of this work was to evaluate if the properties of polyphenolic compound would be maintained, and how long, when added to the milk. For this reason the antioxidant and antiradical activity, and the hydroxytyrosol concentration were measured for seven days.

The results reported here, evidenced how hydroxytyrosol could be usefully added to the milk, without any loss of its antioxidant properties. The importance of the assumption of polyphenol with strong antioxidant properties makes the results promising for further studies on the use in the diet of hydroxytyrosol for application in human health.

Materials and Methods

Polyphenols recovery from olive water

The polyphenolic compound was obtained by the concentration of Olive water, according to [12]. The Olive water was collected in 2014 from olive mill of Antico Frantoio Toscano, Bibbona, Livorno, Italy.

Milk samples

Commercial whole milk was purchased from local supermarket.

The analyses were carried out on milk samples not beyond the expiry date and experiments started as soon as the packs were opened. During the 7 days of experiment the milk samples were stored at 4°C, in the dark. The experiment was carried out in triplicate.

Samples preparation

The solution with polyphenolic compound was prepared at pH 9.0, adjusting the pH with a solution of NaOH. In this study the polyphenolic solution was directly added to the milk considering the final hydroxythyrosol concentration of 50 ± 2 µg/ml, and final pH 6.8, which was the initial pH of the milk alone. The pH was measured at the end of the experiment in order to evaluate if changes occurred.

The adjustment of pH is important to preserve the milk from deterioration and formation of aggregates during the following days.

In order to investigate the distribution of hydroxytyrosol in the milk and its antioxidant and antiradical activity the analyses were carried out on whole milk, whey and casein fraction.

The whole casein fraction was precipitated by adjusting the pH to 4.6, which is its isoelectric point, with drop wise addition of HCl 0.5N [11]. After incubation at 42°C for 20min, the samples were centrifuged at 3000g, at room temperature, for 10min and the supernatants were separated from the pellet, which was constituted by casein. The casein fraction was recovered by adding HPLC-grade water to reach the initial volume. 2ml of the casein solution were mixed with 4ml of HPLC-grade water and 1ml HCl 0.1M. This mix was shaken for 3h, centrifuged at 10000rpm, at 10°C, for 15min. The supernatant was used for analysis and referred to as casein fraction.

As control tests water solution with the same content of treated olive water and milk alone was used.

Total polyphenolic content

The total phenolic content was determined by the Folin-Ciocalteau method [13] using pyrogallic acid (Sigma-Aldrich) as standard. 5ml of Folin-Ciocalteau solution and 10ml of Na carbonate anhydrous (50g/300ml) will be added to 1ml of standard solution and then diluted to 50ml with water. After 2-3 hours in the dark, the absorbance of the solution was spectrophotometrically measured at 730nm. The analysis was carried out in triplicate. The total phenolic content was calculated with the following formula:

g/l Pp = (Abss - Int)/m × dilution, where Abs is the absorption of samples at 730nm after 2 hours, Int the value of the intercept on the x axis of the calibration curve and m the slope of the calibration curve.

Hydroxytyrosol content

To analyze the amount of hydroxytyrosol in the different milk fractions, HPLC analyses were performed according to [14]. For calibration curve, hydroxytyrosol from Carlo Erba was used as standard. Methanol, acetic acid and all solvents used for HPLC were of analytical or HPLC grade from Carlo Erba.

Hydroxytyrosol stock solution was prepared by dissolving the crystalline standard in 1000ppm stock solutions. Subsequently, stock solutions were diluted to 5ppm with 80% methanol (v/v). Calibration curves were obtained for each standard with high linearity ($r > 0.996$) by plotting the standard concentrations as a function of the peak area obtained from HPLC analysis with 25µl injections. For this purpose, the stock solutions of the standards were diluted with 80% methanol to five different concentrations ranging from 1 to 20mg/l. Each concentration was analyzed by triplicate injections.

Analyses of hydroxytyrosol were carried out using a Varian multisolvent pump ProStar 210, a photodiode array detector Varian ProStar 335. For the separations a Phenomenex Kinetex Phenyl-Hexyl 100 A 150 x 4.6 mm reverse-phase C18 column and pre-column operating at 25°C were used. The eluent was composed of (A) H_2O/CH_3COOH (99.9/0.1) and (B) Methanol/H_2O/CH_3COOH (95*/4.9/0.1). A three-step linear solvent gradient system was used starting from 5% to 99% of solution B for a 69-min period at a flow rate of 1.0ml/min. The percentage of solution B reached 25% from 2 to 22 min, then 99% from 23 to 55 min, then 5% from 55 to 69 min. The injection volume was 25µl. UV–Vis spectra were recorded in the 278-325 nm range and the chromatograms were recorded at 278nm.

Antioxidant activity (ORAC Assay (Oxygen Radical Absorbance Capacity))

The method described by [15], was used, using the fluorescence spectrophotometer instrument (Varian Cary Eclipse) (Palo Alto, CA, USA). The sample was added to a free-radical generator (AAPH, 2,2'-azobis (2-aminopropane) dihydrochloride) and the inhibition of the free radical was measured. Fluorescein was used as a target for free radical attack. Free radicals cause conformational changes in the protein structure of fluorescein, leading to dose- and time-dependent fluorescence quenching. The following reagents were added to a quartz cuvette: 2738µl fluorescein (25.5mg/l solution, maintained

Table 1: Characterization of olive water extract used din this experiments.

Composition	Olive water
Polyphenols (g/kg)	25, 22 ± 2.1
Flavonoids (g/Kg)	0, 21 ± 0.01
Hydroxytyrosol (g/kg)	5, 60 ± 0.24
Tyrosol (g/kg)	0, 36 ± 0.02
Phosphate (mg/g)	<0, 04 ± 0.001
Dry weight (g/g)	0, 767 ± 0.031
Carbohydrates (g/kg)	45, 84 ± 2.83
Proteins (g/kg)	52, 31 ± 2.55

at 4°C), 37µl phosphate buffer solution (75mM, pH 7.4) and 150µl Trolox standard (Sigma-Aldrich, 20µm), blank (buffer solution) or sample solution. After incubation at 37°C for 30min, the addition of 75µl AAPH solution (86.8mg/ml in buffer solution and kept in ice) started the reaction. The exciting λ is 490 nm and the emission λ is 512nm. Total antioxidant capacity or ORAC unit (µm) is given by the following formula:

ORAC value = 20 k (ASample - Ablank) / (ATrolox - Ablank) x [Trolox]/[Sample] with k the dilution factor, Asample the under curve area of the sample, Ablank the under curve area of the blank and ATrolox™ the under curve area of the standard.

Antiradical activity (DPPH radical scavenging assay, RSA)

DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich) is a stable radical that can be reduced by reaction with an antiradical hydrogen-donor compound. The method based on the procedure of Brand-Williams was used [16]. This colorimetric reaction is measured with a spectrophotometer (Varian Cary Eclipse) at 517nm: the DPPH radical color shifts from violet to yellow. The sample was diluted as necessary in ethanol. 1ml of diluted extract was added to 1ml of ethanolic DPPH solution (63µm) and mixed and measured immediately. The absorbance was measured again after 20 minutes. Different dilution was tested in order to find the concentration needed to reduce by 50% the DPPH free radicals.

A decrease by 50% of the initial DPPH concentration is referred as IC_{50} that is the inhibition concentration by 50% of DPPH. Due to the green dark color of the extracts the absorbance value at 517nm of 1ml each sample at the same dilution of the analysis, added to 1ml of ethanol was subtracted to all the absorbance values for each samples.

For each extract the IC_{50} was calculated with the following formula:

% inhibition = [100-(Ax/As)] x 100

where as is the initial absorbance of the sample extract in DPPH solution (t = 0) and Ax the absorbance of the same sample after 20 minutes (t 20min).

At least 4 different concentration of the extracts were used to determinate the IC_{50}.

Microbiological test

The total viable microflora count in the milk sample was measured by pour-plate method using plate count agar (Merck, Darms-tadt, Germany). The plates were incubated at 30°C for 168h. Measurements were carried out after 72h and 168h. Data are expressed as $\log_{10}$ of colony forming units (CFU)/ml [10].

Results and Discussion

Olive water

The polyphenolic sample recovered by Olive water was characterized, in order to evaluate quantitatively and qualitatively the polyphenolic content. The results are reported in Table 1.

The most relevant result was the polyphenolic content, and particularly the high hydroxytyrosol content.

pH of enriched milk and casein and whey fraction in the sample

When polyphenols were added to the milk, the pH was adjusted, as reported in the section materials and methods, in order to have in all the samples the same initial pH of the milk. In the enriched milk the value of pH 6.8, within the 7 days, showing a slight increase to pH 7.0. In the milk alone and in the control the pH after 7 days changed, being 6.35 and 7.25, respectively (Table 2). The results indicated that the polyphenol addition to the milk may have preserved the milk by the bacterial contamination, which occurred in the milk alone, inducing a pH decrease for the production of lactic acid [17].

These data were in accordance with previous findings obtained by adding oleuropein to milk and yogurt [10]. Oleuropein, a polyphenol present in olive fruit, has a high bioactive activity, and its addition to milk led to pH 6.45 and it did not changed after storage at 4°C for 7 days.

Looking at the different components of the milk, the results showed that at the beginning of the experiment the casein constituted 40% of the milk, whereas the whey was 60% of the total volume (Figure 1). After 48h, and during all the following period of the experiment, the casein fraction slightly increased to 45.

This results indicated that the polyphenols added to the milk, maintaining the initial pH, could preserve the milk by degradation, and alteration of the different component.

Table 2: Changes in pH value and microbial analysis of Control (C), milk enriched in polyphenols (P milk) and control milk (C milk) and milk with addition of ethanol (Et milk).

Samples	Start	72h	168h	Start	72h	168h
	pH			Log_{10} of CFU/ml		
C	6.80 ± 0.01	6.95 ± 0.02	7.25 ± 0.05	-	2.08 ± 0.50	2.83 ± 0.10
P milk	6.80 ± 0.02	6.87 ± 0.03	7.00 ± 0.07	-	2.85 ± 2.50	3.22 ± 0.02
C milk	6.80 ± 0.02	6.54 ± 0.02	6.35 ± 0.03	-	2.81 ± 0.05	> 5
Et milk	6.51 ± 0.02	6.54 ± 0.04	6.55 ± 0.02	-	2.75 ± 0.04	2.81± 0.06

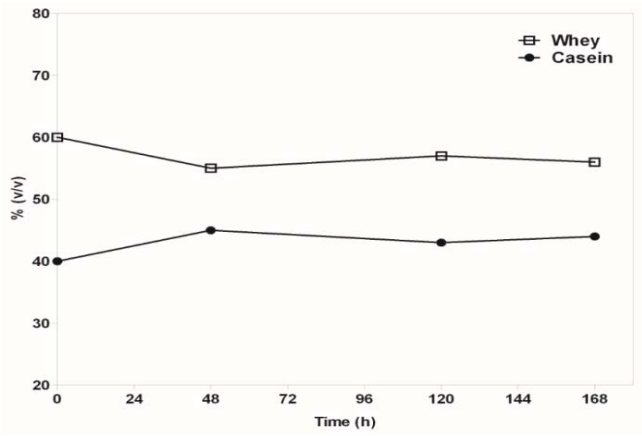

Figure 1: Changes in whey (open square) and casein (closed circle) fraction in the milk enriched in polyphenols from olive water. The results are expressed as % of each fraction on the total volume of the milk.

Microbiological analysis

The measurements of bacterial population of the milk enriched with hydroxythyrosol are reported in Table 2. The resulted showed that within 48 hours the cfu/ml were of the same extent both in the control sample and in the enriched milk, 2.81 and 2.85 log cfu/ml, respectively. After 7 days the microflora in the milk alone resulted larger than in the enriched milk, being more than 5 and 3.22 log cfu/ml, respectively.

This result may reflect the positive influence of the hydroxytyrosol addition in the milk, on the growth of microflora constitutively present once package has been opened. This is in accordance with the changes in pH values in the milk alone, which were not evidenced in the enriched milk, and with previous findings which reported that the addition of polyphenols to food can be considered as a tool for preservation of food from deterioration [18].

In this contest the aim of the work was to monitor the antioxidant and antiradical activity of polyphenolic compound recovered from olive water, when added to the milk, and to establish if this activity was maintained during the following days.

Distribution of polyphenols content in the milk

The polyphenols content added to the milk did not exhibited strong changes during the analyzed period of 7 days (Table 3).

Concerning the distribution of polyphenols in the milk, it is important to take into account the different fraction occupied by the whey and casein on the total volume of the milk.

Looking at the polyphenols distribution in the milk it resulted evident that the polyphenolic extract was not equally distributed between the whey and casein fraction.

It is interesting to observe that the whey exhibited a concentration of total polyphenols which represented the 65% of the total amount, while the casein fraction alone, and contained the 35%. This trend was maintained for all the 7 days (Table 3).

These results may seem to be in contrast with previous findings, showing the formation of complex between polyphenols of tea, catechins, and the casein [19]. In our case, the nature of polyphenols was different from the one in the tea. The polyphenolic extract used for these experiments was mainly constituted by hydroxytyrosol, which is one of the most active antioxidant polyphenolic compounds present in the olive water.

Table 3: Changes in total polyphenolic (Ptot) and hydroxythyrosol (OH-Tyr) content in the control (C), in milk enriched in polyphenols (P milk), and different compartment of the enriched milk: whey (P whey) and casein (P casein) fraction.

Samples		start	48h	120h	168 h
	mg/ml				
C	Ptot				
	OH-Tyr	48 ± 1	45 ± 0.8	45 ± 1	42 ± 2
P milk	Ptot	1.74 ± 0.09	1.55 ± 0.07	1.53 ± 0.06	1.55 ± 0.06
	OH-Tyr	49 ± 2	41 ± 1	42 ± 2	36 ± 1
P whey	Ptot	1.10 ± 0.05	1.12 ± 0.06	1.02 ± 0.04	1.11 ± 0.05
	OH-Tyr	40 ± 1	35 ± 0.9	32 ± 1	29 ± 0.5
P casein	Ptot	0.59 ± 0.03	0.48 ± 0.02	0.41 ± 0.01	0.53 ± 0.03
	OH-Tyr	9 ± 0.5	10 ± 0.7	7 ± 0.8	8 ± 0.6

Table 4: Change sin antioxidant activity in the control (C), in milk enriched in polyphenols (P milk), and different compartment of the enriched milk: whey (P whey) and casein (P casein) fraction. Changes in control milk (C milk) and milk with addition of ethanol (Et milk) are reported.

Samples	start	48h	120h	168h
	μM TE/ml			
C	7.370 ± 0.5	7.250 ± 0.3	6.470 ± 0.3	5.430 ± 03
P milk	17.212 ± 0.8	15.314 ± 0.6	13.915 ± 0.8	12.46 ± 0.6
P whey	21.656 ± 0.9	20.984 ± 1.2	20.702 ± 1.2	20.28 ± 1.1
P casein	6.827 ± 0.5	6.653 ± 0.4	6.340 ± 0.4	6.170 ± 0.5
C milk	8.180 ± 0.4	7.020 ± 0.5	6.840 ±0.5	5.780 ± 0.4
Et milk	2.630 ± 0.2	2.510 ± 0.1	2.070 ± 0.2	2.530 ± 0.1

Its content in the milk and its distribution both in the whey and casein was monitored during the 7 days. It resulted evident that from the beginning most of the hydroxytyrosol was distributed in the whey, 82%, while only 18% was found in the casein fraction (Table 3). This trend was maintained for all the 7 days, with a slight decrease to 79% in the whey at the 7th day, and to 21% in the casein.

Antioxidant activity (ORAC)

The antioxidant activity in the milk with the addition of polyphenolic extract was maintained for all the 7 days (Table 4). The antioxidant activity exhibited by the milk was in accordance to what previous reported [11] and resulted lower than the polyphenolic enriched milk, indicating that the polyphenols addition conferred antioxidant properties to the milk. In particular, the increment of the antioxidant activity in the supplemented milk resulted of the same extent of the one of the polyphenolic extract, reported as control (Table 4). These results are very interesting, as they indicated that the polyphenolic compounds added to the milk did not encounter any inhibition in explicating their antioxidant activity. Previous findings reported the reduction of antioxidant properties of polyphenols, catechins of green tea, by the addition of milk [20]. In our experiments, interestingly, the antioxidant activity in the whey resulted more

than 3-fold higher than in the casein fraction. As showed by the polyphenols distribution in the milk, the hydroxytyrosol added to the milk was mainly found in the whey. However, this result may be explained by the fact that hydroxytyrosol has a size smaller that the catechin one and a lower number of hydroxylic groups. Indeed, in the case of the catechins the inhibition of antioxidant activity by the milk was attributed to the formation of complexes between catechins and milk globular proteins, in particular β-lactoglobulin (β-LG) [21]. It is known that the capability of polyphenols to bind proteins is size dependent [22] increasing with the molecular size. The binding has been proposed to inhibit the capacity of catechins to reduce the hydroxyl groups by donation of electrons. In the case of hydroxytyrosol the results indicated that its activity was not inhibited by binding to the β-LG. Other authors reported the casein as the main milk protein involved in the binding and responsible of decreasing the properties of polyphenolic compounds in tea [19]. However, in our case, the results showed that the casein fraction did not contain a large amount of hydroxytyrosol, so the interaction of hydroxytyrosol with all the milk protein did not occur and no effect on its action could be detected.

These results pointed out how hydroxytyrosol added to the milk could explicate its antioxidant activity mainly in the whey fraction, in accordance to the main presence in this compartment.

It could be observed that the antioxidant activity of the whey alone was higher than the one of the milk.

It has been measured an antioxidant capacity in cow milk, which has been mainly ascribed to the casein fraction [12]. This capability of casein could be increased by adding tea polyphenols, catechins, as already reported, and forming micelles acting as vectors of antioxidants [7,19].

The results presented here, pointed out how, with the addition of hydroxytyrosol, which mainly distributes in the whey, the milk acquires a broader spectrum of action, increasing the bioavailability of polyphenols added to the milk.

Antiradical activity (DPPH)

Table 5 reported results on the radical activity. The extract maintained its initial activity of 0.132ml for 48h but after 120h the amount for scavenging 50% DPPH free radical was 10-fold higher. Interestingly, the extract added to the milk maintained almost all its initial antiradical activity of 0.143ml (Table 5), exhibiting only a partial increase (by 30%) after 48h, which remained the same for all the 7 days.

Looking at the antiradical activity of the different components of the enriched milk, it resulted evident that at the initial time, most of the activity to scavenge the free radicals was performed by the whey, the amount of which was 7-fold lower than the one of the casein fraction (Table 5). This trend could be observed for all the 7 days, during which the casein fraction did not change its activity, whereas the whey fraction increased its antiradical activity by 55% (Table 5). The highest capability of the whey fraction to reduce by 50% the free radicals, in the DPPH reaction assay, confirmed what observed for the antioxidant activity.

These results reflected the hydroxytyrosol distribution in the milk, more concentrated in the whey than in the casein.

Table 5: Changes in antiradical activity in the control (C), in milk enriched in polyphenols (P milk), and different compartment of the enriched milk: whey (P whey) and casein (P casein) fraction. Changes in control milk (C milk) and milk with addition of ethanol (Et milk) are reported.

Samples	Start	48 h	120 h	168 h
	ml to reduce by 50% the DPPH radicals			
C	0.132 ± 0.02	0.114 ± 0.005	1.455 ± 0.06	1.263 ± 0.05
P milk	0.143 ± 0.01	0.100 ± 0.004	0.094 ± 0.01	0.107 ± 0.01
P whey	0.096 ± 0.004	0.035 ± 0.001	0.061 ± 0.02	0.043 ± 0.002
P casein	0.682 ± 0.03	0.742 ± 0.02	0.510 ± 0.03	0.582 ± 0.03
C milk	1.207 ± 0.05	1.304 ± 0.07	1.279 ± 0.05	1.567 ± 0.05
Et milk	2.052 ± 0.12	1.931 ± 0.10	1.558 ± 0.06	1.571 ± 0.09

The milk and the milk added with ethanol exhibited a lower activity, with respect to the milk with addition of extract (Table 5).

These results showed that the antiradical activity of the polyphenolic extract was preserved when added to the milk, in accordance to what observed for the antioxidant activity.

Organoleptic characteristics

From the organoleptic point of view the adding of polyphenols changed the enriched milk, which resulted changed in a slightly brown, color, with a slightly bitter taste. Although the taste of this milk was acceptable, this aspect has to be taken into account. Some solutions have been proposed, considering the possibility to encapsulate these compounds [9] or different procedure in the treatment of olive water.

Further studies will be undertaken in order to ameliorate the hydroxythyrosol enriched milk taste for a best consumption.

Conclusion

The use of antioxidant natural compounds is attracting an increasing interest. Moreover, addition of antioxidants in food may prevent the deterioration due to the lipid oxidation [4].

The results presented here suggest a new possible role for milk, in addition to the one proposed for the formation of complexes with casein and tea catechins, against colon cancer cells [7]. In the case of hydroxytyrosol, which was mainly found in the whey fraction, a different role of milk, as vehicle of bioactive compounds, can be hypothesized.

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