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ORIGINAL CONTRIBUTION

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Antioxidant effectiveness of organically and non-organically grown red oranges in cell culture systems

Summary *Background* Consumers consider plant food products from organic origin healthier than the corresponding conventional plant foods. Clear experimental evidence supporting this assumption is still lacking. *Aim of study* To determine if the organic red oranges have a higher phytochemical content (i. e., phenolics, anthocyanins and ascorbic acid), total antioxidant activity and *in vitro* bioactivity, in terms of protective effect against oxidative damage at cellular level, than non-organic red oranges. *Methods* Total phenolics were measured using the Folin Ciocalteau assay, while total anthocyanins and ascorbic acid levels were determined by spectrophotometric and HPLC analysis, respectively. In addition, the total antioxidant activity of red orange extracts was measured by the $ABTS^{\cdot+}$ test. The ability of red orange extracts to counteract conjugated diene containing lipids and free radical production in cultured rat cardiomyocytes and differentiated Caco-2 cells, respectively, was assessed. *Results* Organic oranges had significantly higher total phenolics, total anthocyanins and ascorbic acid levels than the corresponding non-organic oranges (all $p < 0.05$). Moreover, the organic orange extracts had a higher total antioxidant activity than non-organic orange extracts ($p < 0.05$). In addition, our results indicate that red oranges have a strong capacity of inhibiting the production of conjugated diene containing lipids and free radicals in rat cardiomyocytes and differentiated Caco-2 cells, respectively. Statistically higher levels of antioxidant activity in both cell models were found in organically grown oranges as compared to those produced by integrated agriculture practice. *Conclusions* Our results clearly show that organic red oranges have a higher phytochemical content (i. e., phenolics, anthocyanins and ascorbic acid), total antioxidant activity and bioactivity than integrated red oranges. Further studies are needed to confirm whether the organic agriculture practice is likely to increase the antioxidant activity of other varieties of fruits and vegetables.

Key words red orange – integrated agriculture – organic agriculture – phenolics – antioxidant activity

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Introduction

The growing demand for organic plant foods can largely be attributed to concerns about environmental protection and food safety (including the effects of exposures to pesticide residues and other synthetic chemicals), along with interest in the relationship between diet and health. Organic agriculture procedures have become one of the fastest growing sectors of North American and European agriculture [1, 2]. According to European Community Regulations (2092/91/ECC and updatings), 'organic' plant foods are those produced without the use of synthetic chemical pesticides and largely without the

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addition of readily soluble mineral fertilizers. It is thought that in the absence of pesticides, plants could contain higher levels of antioxidant components as a result of enhanced synthesis of active phytochemicals produced in defence against biotic and abiotic stress [3, 4]. A recent systematic review [5] tentatively suggested that organic produce could contain 10–50 % more phytochemicals than non-organic produce. Although organic produce is often compared with produce from cultivations based on the use of synthetic fertilizers and pesticides, within the European Community countries these so-called 'conventional' methods have been officially replaced by 'integrated' systems that reduce the use of chemicals by combining organic and conventional techniques [6].

The health-related properties of the phenolic compounds contained in fruit largely derive from their antioxidant activity [7, 8]. Red oranges (so-called 'blood' oranges) provide a particularly rich natural source of ascorbic acid and phenolic compounds, including anthocyanins (the abundant presence of red anthocyanin pigments accounts for the characteristic ruddy appearance of these Moro, Tarocco and Sanguinello varieties [9, 10]). Rapisarda et al. [11] showed that anthocyanin content is the main factor influencing the antioxidant activity of pigmented orange fruit. Furthermore, a considerable body of evidence indicates that the anthocyanin phenolics present in red orange fruit can play a key role in the prevention of human pathologies related to oxidative damage of biomolecules, including heart diseases and cancer [12]. The concentration of anthocyanins in oranges can depend on a wide range of variables, including genetic and physiological factors, the soil, climate and ripening conditions [13, 14]. Comparisons of concentrations of phenolic compounds in red oranges grown by integrated and organic agricultural practices are currently lacking.

We determined total phenolics, total anthocyanins, ascorbic acid levels and total antioxidant activity in red oranges grown by integrated and organic agricultural practices. We then examined bioactivity of these oranges in terms of protective effect against oxidative damage at cellular level. Two different cell models were used: (i) primary cultures of neonatal rat cardiomyocytes, and (ii) a human colon carcinoma cell line (Caco-2). The rat cardiomyocytes allow assessment of the lipid peroxidation which is typical of myocardiac ischemic damage [15]. Caco-2 cells differentiated to normal intestinal epithelia [16] provide a suitable model for assessment of the physiological response of intestinal epithelia to oxidative injury [17, 18].

Materials and methods

Chemicals

Gallic acid, vitamin C, trypan blue, fetal calf serum (FCS), horse serum (HS), tert-butyl hydroperoxide (*t*-BuOOH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2',7'dichlorodihydrofluorescein diacetate (DCFH-DA) and Folin-Ciocalteu's phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade purity commercially available.

Sample selection

Red oranges ('Tarocco' cultivar) harvested in October-November 2003 from certified producers operating in eastern Sicily (Italy) and applying either integrated or organic agricultural practices were purchased from a single retail outlet (all the integrated/organic fruits were individually labeled as products produced under controlled cultivation conditions in line with the provisions of statutory European Community regulations regarding integrated and organic farming). Four different lots of six fruits at comparable length commercial periods from harvest time were analyzed for either type of agricultural practice. All fruits have been chosen according to their similar size and weight. No physical defects or signs of pathogen contaminations were evidenced. All fruits were processed within 48–72 h of purchase.

Sample extraction

The samples were processed as reported by Sun et al. [19]. Briefly, samples of 100 g of the pooled fresh edible part of each lot of fruits were homogenized with chilled 80 % acetone (1:2 w/v) using a chilled Waring blender for 5 min. The homogenates were filtered through a Whatman paper under vacuum, and the acetone in the filtrate was evaporated at 45 °C until approximately 90 %. The filtrate was than diluted to 50 ml with water and stored at –20 °C until use.

Total phenolic content analysis

Total phenolic concentrations were measured using the Folin Ciocalteau assay [20]. Briefly, appropriate dilutions of extracts were treated with Folin Ciocalteau reagent and the reaction was neutralized with 7 % sodium carbonate. The absorbance of the resulting blue color was measured spectrophotometrically at 750 nm using a Beckman DU 7400 spectrophotometer. Gallic acid was used as standard and results expressed as milligrams of

gallic acid equivalents per 100 g of fresh edible part of the fruits. Due to the contribution of ascorbic acid to the absorbance measurement in the Folin Ciocalteau assay, ascorbic acid levels were evaluated separately by reversed-phase high-performance liquid chromatography (RP-HPLC) (see below). The absorbance of the known amounts of ascorbic acid, corresponding to the values measured in the samples, was determined in the Folin Ciocalteau assay, and values were deducted from the spectrophotometrically determined total phenolics value as suggested by Asami et al. [21].

Total ascorbic acid analysis

Analysis of total ascorbic acid levels in the edible parts of integrated and organic red oranges was carried out by RP-HPLC as reported by Lee and Chen [22]. Samples (10–20 g) were homogenized with 3% metaphosphoric acid/acetic acid (85:15 v/v) at 1:5 (w/v) ratio for 1 min. The homogenate was filtered and diluted to appropriate concentrations with 2% KH_2PO_4 (pH 2.4). RP-HPLC analysis was performed using a Waters model 600E system controller, a Waters model 717 plus autoinjector (Waters Corp., Milford, MA), a Spectra Physics model 200 programmable wavelength UV detector and a Zorbax C18 ODS2 column (5 μm, 250 × 4.6 mm). Ascorbic acid was eluated isocratically using 2% KH_2PO_4 (pH 2.4) as mobile phase at a flow rate of 0.5 ml/min and the eluate was monitored at 245 nm. Integration was performed using the Millennium chromatography software from Waters Corp. The calibration curve was obtained using pure ascorbic acid.

Total anthocyanin content analysis

Total anthocyanin content of the extracts of integrated and organic red oranges was then determined spectrophotometrically by the pH differential method of Rapisarda et al. [23].

Total antioxidant activity

The total antioxidant activity of the extract derived from integrated and organic red oranges was measured as reported by Re et al. [24]. This method is based on the ability of the antioxidant molecules in the fruit extracts to reduce the radical cation of the ABTS, determined by the decolorization of $ABTS^{\cdot+}$ and measured as quenching of absorbance at 740 nm. Values obtained for each sample were compared with the concentration-response curve of a standard Trolox solution, and expressed as Trolox Equivalent Antioxidant Activity (TEAA)/100 g of fresh fruit.

Cultures of neonatal rat cardiomyocytes and protection by hypoxia/reoxygenation injury

Primary heart cell cultures were obtained by isolation of cardiomyocytes from the ventricles of 2 to 4 day old Wistar rats, as previously reported [15]. Cells were grown until confluence in 60 mm Petri dishes containing nutrient mixture Ham F10 supplemented with 10% v/v FCS and 10% v/v HS. Then, 24 h before the hypoxic period, cardiomyocytes were supplemented with different concentrations of the extracts derived from integrated and organic red oranges corresponding to 12.5-100 mg fruit/ml. Before starting the experiments, the medium was removed, and cells were washed with phosphate buffered saline (PBS) and incubated in F10 medium without serum, which had been pregassed with bubbling 95% nitrogen-5% CO_2 in order to achieve near anoxic conditions. In cells previously supplemented with the extracts, the same concentrations of the extracts were maintained in this hypoxia medium. The culture plates were then transferred to specially designed, airtight, thermostated chambers for a 4 h hypoxia phase prior to reoxygenation. At the beginning of the 1 h reoxygenation period, the hypoxia medium was changed to Ham F10 medium supplemented with 10% HS and 10% FCS and, in cardiomyocytes pretreated with the extracts, with the same concentrations of the extracts themselves.

The hypoxic procedure reduced oxygen from 20 to 5% after 3 min, and to 1% after 10 min. The O_2 content of the atmosphere inside the chamber was < 1% for the duration of the experiment, as measured by an on-line meter (Griffin and George, Fife, UK) [25]. Reoxygenation increased oxygen to 20% within 5 min. At the end of the experiments, the appearance of conjugated diene-containing lipids was evaluated as an index of lipid peroxidation using the method described by Burton et al. [26]. Briefly, cells scraped from the culture plates were extracted in chloroform:methanol:water (2:1:1 v/v). The chloroform layers from two extractions were combined and then dried under nitrogen. Samples were resuspended in a known volume of acetonitrile and absorbance determined at 235 nm.

Cultures of human colon carcinoma cells and protection by oxidative injury

Human colon carcinoma (Caco-2) cells were routinely grown at 37 °C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (FCS), 2 mmol/L glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. To evaluate intracellular antioxidant and cytoprotective activity of the red orange extracts, Caco-2 cells were seeded at a density of 8×10^4 cells/cm^2 in multi-well dishes; once the

cells were confluent, the medium was changed every 48 h using an DMEM 20% FCS. Experiments were performed using completely differentiated cultures at 12–14 days post seeding.

We evaluated the intracellular antioxidant and cytoprotective activities of red orange extracts against both formation of intracellular reactive oxygen species (ROS) and cytotoxicity in differentiated Caco-2 cells after treatment with *t*-BuOOH, a compound used to induce oxidative stress.

Formation of intracellular ROS was determined using a fluorescent probe, DCFH-DA, as described by Wang H. et al. [27]. Briefly, differentiated Caco-2 cells were incubated for 24 h with different concentrations of the extracts derived from integrated and organic red oranges corresponding to 6.25–50 mg fruit/ml. Cells were washed with PBS and then incubated with 5 μM DCFH-DA in PBS in 5% CO_2 at 37 °C for 30 min. After removal of DCFH-DA and further washing, the cells were incubated with 3 mM *t*-BuOOH in PBS for 1 h. At the end of incubation, the fluorescence of the cells from each well was measured (wavelength; 485/535 nm) with a spectrofluorometer (Spectra Max Gemini, Molecular Devices, Minnesota). Intracellular antioxidant activity was expressed as percentage inhibition of intracellular ROS evoked by exposure to *t*-BuOOH.

Cytotoxicity was monitored by trypan blue uptake as previously described [28]. Briefly, differentiated Caco-2 cells were incubated for 24 h with extracts of red orange (corresponding to 6.25–50 mg fruit/ml), washed with PBS and then incubated with 3 mM *t*-BuOOH in 5% CO_2 at 37 °C. After 3 h of incubation, the cells were collected by gentle scraping in PBS and dispersed by repeated gentle pipeting. An aliquote of cell suspension was then diluted 1:1 with 0.5% trypan blue in 10 mM sodium phosphate buffer (pH 7.2) and placed on a hemocytometer with a cover slip. Percentages of stained cells were recorded on at least three separate counts. Cytoprotective activity was expressed as percentage inhibition of cytotoxicity evoked by exposure to *t*-BuOOH.

Statistical analysis

Data are reported as mean ± SD. Statistical analysis was performed using the Student's *t*-test for comparisons of means and Pearson's correlation coefficient for relations among variables. Values were considered significant with $p < 0.05$. Analyses were performed using STATISTICA 4.5 run on Windows.

Results

We determined the phytochemical content such as phenolics, anthocyanins and ascorbic acid in red oranges grown by integrated and organic agricultural practices. As reported in Table 1, organic oranges had a significantly higher phenolic, anthocyanin and ascorbic acid content than the corresponding integrated oranges (all $p < 0.05$).

Subsequently, we determined the total antioxidant activity of fresh red oranges. Extracts from the two different cultivation types were submitted to the ABTS radical cation decolorization assay and the activities of the extracts expressed as μmol of Trolox Equivalents/100 g edible pulp. On the basis of fresh weight, the total radical scavenging ability of the organic oranges was significantly higher than the activity of integrated oranges (741 ± 24 vs 669 ± 31 μmol of Trolox Equivalents/100 g edible pulp, $p < 0.05$).

To investigate bioactivity, we first examined the integrated and organic red orange's protective effect against oxidative damage in primary cultures of neonatal rat cardiomyocytes.

Both integrated and organic orange extracts showed a marked antioxidant activity in cultured cardiomyocytes in which oxidative stress was induced by hypoxia/reoxygenation. As shown in Fig. 1, treatment of cardiomyocytes with both integrated and organic orange extracts decreased the production of conjugated diene lipids (as an index of lipid peroxidation) after 4 h hypoxia followed by 1 h reoxygenation, although to a different extent, according to the extract concentration. Conjugated diene levels decreased proportionally with the increase of the concentration of the extracts, and the correlation between the concentration and the entity of conjugated diene levels was statistically significant for both the integrated ($r = -0.981$, $p < 0.01$) and the organic orange extracts ($r = -0.969$, $p < 0.01$). Although conjugated diene levels in the cultures treated with the organic extract were always lower than those measured in cultures treated with the integrated orange extract, only the highest concentrations used (50 to 100 mg/ml) revealed a significant difference ($p < 0.01$) in the ability of integrated and organic orange extracts to counteract

Table 1 Total phenolics, Total ascorbic acid, Total anthocyanins of fresh red orange[1]

Agricultural practice	Total phenolics[2]	Total ascorbic acid	Total anthocyanins
	mg of GAE/ 100 g orange	mg of ascorbic acid/ 100 g orange	mg of anthocyanins/ 100 g orange
Integrated	74.0 ± 3.0	45.0 ± 4.0	5.56 ± 0.42
Organic	79.0 ± 3.0*	53.0 ± 4.0*	6.65 ± 0.35*

[1] Values are means ± SD of at least four different lots (organic vs. integrated; *$p < 0.05$)
[2] Values were expressed as gallic acid equivalents (GAE) in milligrams per 100 g of fresh edible part of red orange

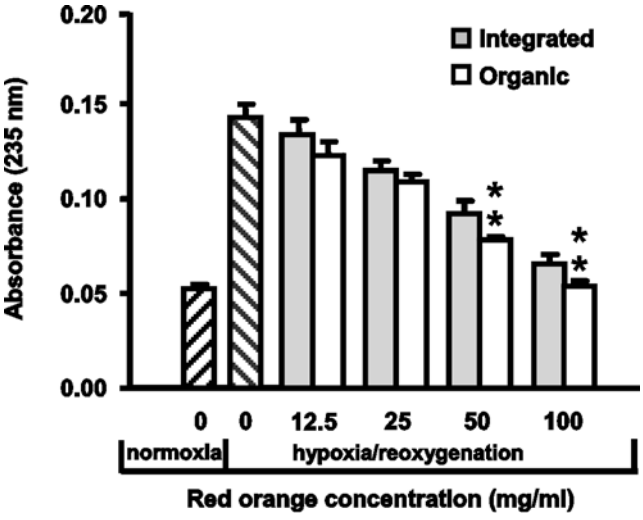

Fig. 1 Conjugated diene production in cardiomyocytes after 4 h of hypoxia and 1 h reoxygenation in the absence or presence of integrated and organic extracts. Some cardiomyocytes were supplemented with integrated and organic red oranges extracts 24 h prior to the experiment, then subjected to hypoxia in the presence of the same antioxidants. Conjugated diene production was measured as 235 nm absorbance as reported under the Methods. Values are means ± S. D. of at least three different cell cultures. (organic vs integrated, **p < 0.01)

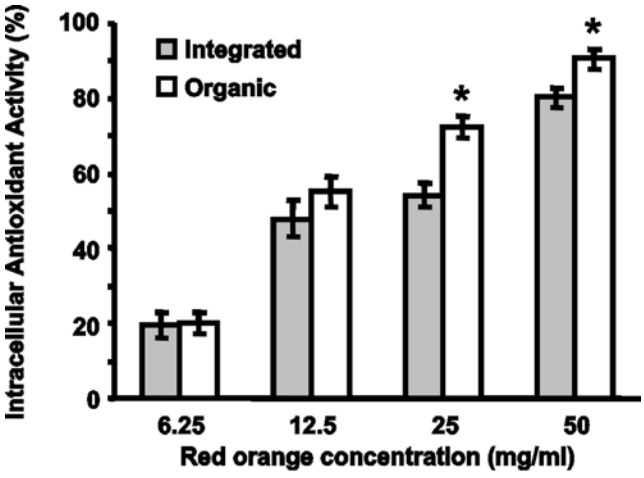

Fig. 2 Intracellular antioxidant activity of red oranges from integrated and organic cultivars in Caco-2 cells differentiated to normal intestinal epithelia. Levels of intracellular antioxidant activity are expressed as the percentage of inhibition of intracellular ROS evoked by exposure to *t*-BuOOH after incubation for 24 h with red orange extracts. Values are means ± S. D. of at least three different cell cultures (organic vs integrated, *p < 0.05)

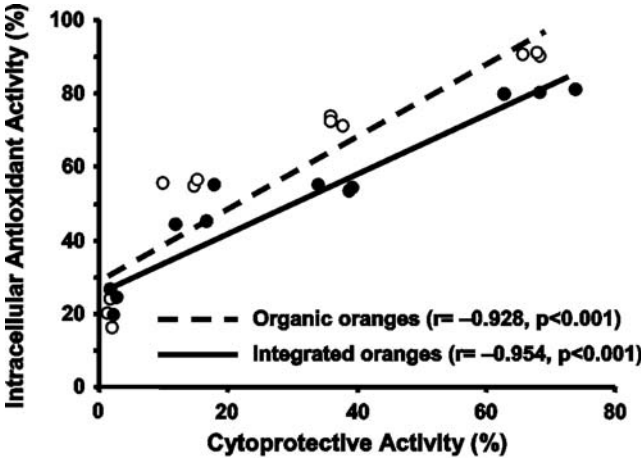

Fig. 3 Correlation between intracellular antioxidant and cytoprotective activities of red oranges from integrated and organic cultivars in Caco-2 cells differentiated to normal intestinal epithelia. Levels of intracellular antioxidant and cytoprotective activities are expressed as inhibition percentages of intracellular ROS and cytotoxicity, respectively, evoked by exposure to *t*-BuOOH after incubation for 24 h with red orange extracts

lipid peroxidation; treatment of cardiomyocytes with the highest concentration of the organic orange extract (100 mg/ml) completely prevented the appearance of conjugated diene lipids.

We then assessed the bioactivity of red orange extracts in terms of the intracellular antioxidant and cytoprotective activities against both formation of ROS and cytotoxicity in differentiated Caco-2 cells after treatment with *t*-BuOOH, as the control compound used to induce oxidative damage. As shown in Fig. 2, treatment of differentiated Caco-2 cells with both integrated and organic orange extracts (6.25 to 50 mg/ml) showed a marked dose-dependent increase of intracellular antioxidant activity. At 25 to 50 mg/ml extract, antioxidant activity was significantly higher with organic than integrated orange samples (both $p < 0.05$). Interestingly, a highly significant linear correlation was found between antioxidant activity and cytoprotective activity for both the integrated ($r = -0.954$, $p < 0.001$) and the organic ($r = -0.928$, $p < 0.001$) orange extracts (Fig. 3). However, despite a higher antioxidant activity in organic than integrated orange extracts, no significant differences in the cytoprotective activity between organic and integrated samples were observed (Fig. 4).

Discussion

In the frame of a national project aimed at the characterization and valorization of typical Italian organic plant foods, we have compared the phytochemical contents and antioxidant effectiveness of integrated and organic red oranges ('Tarocco' cultivar) of Sicily, a traditional food of the Italian diet.

Our results show that fresh organic red oranges have significantly higher total phenolic and ascorbic acid levels than the corresponding non-organic oranges. This finding is consistent with a general improvement in the antioxidant system developed by the plant in organic fruits [29, 30]. In fact, phenolic compounds are likely to

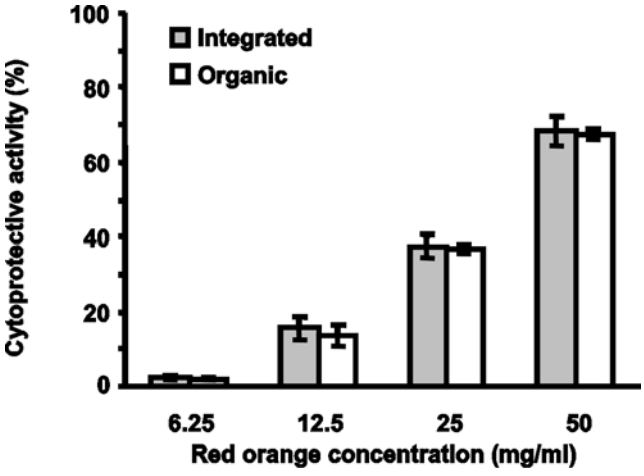

Fig. 4 Cytoprotective activity of red oranges from integrated and organic cultivars in Caco-2 cells differentiated to normal intestinal epithelia. Levels of cytoprotective activity are expressed as the percentage of inhibition of cytotoxicity evoked by exposure to *t*-BuOOH after incubation for 24 h with red orange extracts. Values are means ± S. D. of at least three different cell cultures

be involved in the plant defense mechanism by acting as a chemical barrier to invading phytopathogens, and modulation of the levels of these compounds by pesticides (herbicides, insecticides and fungicides) has been observed [3, 4]. Available data on the effect of cultivation system on ascorbic acid content of fruits and vegetables gave controversial results [31]. These results are difficult to interpret because the growing conditions varied widely and different methods of sampling and analysis have been used. Ascorbic acid content was increased in the organic as compared with conventional peach, but not pear fruit samples [29]; unfortunately, data on the comparison between integrated and organic oranges are not available. However, the amount of ascorbic acid determined in our red orange samples is consistent with other published values for the 'Tarocco' cultivar [32].

The pigmented cultivar 'Tarocco' differs from the blond orange group by the presence in the flesh, and sometimes in the rind, of red pigments belonging to anthocyanin classes [33]. Interestingly, total anthocyanin content in our organic oranges was significantly higher than in integrated oranges.

The concentration of anthocyanins can be dependent on genetic and physiological factors, on the ripening of the fruits, on the soil and climate characteristics and on agricultural practices [13, 14]. Notwithstanding these limitations, the amount of anthocyanins found in our samples of red oranges are fully comprised in the range of values already published for the 'Tarocco' cultivar [34].

In parallel with these findings, a higher increase of total antioxidant activity of organic than non-organic oranges was also detected. The antioxidant activities of oranges measured with the ABTS assay were quite consistent with those measured with the oxygen radical absorbance capacity (ORAC) assay [35]. Red oranges are a good nutritional source of vitamin C; nevertheless, since the antioxidant capacity of 1.0 μmol of vitamin C is 0.99 Trolox equivalents [36], the amount in the samples, both organic and integrated, may account for only 30–40 % of the total antioxidant activity of the fruits. These findings confirm that phenolic molecules may be important antioxidant components to account for the observed activity.

To our knowledge, this is the first time that the bioactivity of red oranges has been studied with respect to different agriculture practices. Our results indicate that red oranges have a strong capacity of inhibiting the production of conjugated diene lipids and free radicals (two markers of oxidative damage at cellular level) in rat cardiomyocytes and differentiated Caco-2 cells, respectively. In addition, organically grown oranges exibited statistically significant higher antioxidant activity than the non-organically grown oranges, in both cell models. It is interesting to note how the major concentrations of ascorbic acid (18 %) and anthocyanins (20 %) determined are related to a major antioxidant activity of organic orange extracts. By contrast, antioxidant effectiveness of organic cultivar did not appear to translate into any significant increase in the cytoprotective activity. Most probably, the antioxidant content in organic orange samples extracts is not high enough to increase the ability to counteract the cytotoxicity, as a marker of cumulative oxidative damage to cellular constituents.

Further studies are needed to confirm whether the organic agriculture practice is likely to increase the antioxidant activity of other varieties of fruits and vegetables. Moreover, relatively few scientific data are available on the possible health-related properties of organic fruits, and it is not known whether and to what extent these products actually provide health advantages for the human population [37–40].

Our data clearly showed that red oranges have potent antioxidant activity and exhibit cytoprotective effects independently of their growth methods. Therefore the supplementation of natural antioxidants through a balanced diet rich in red oranges could be an effective and also economic way to increase resistance of biomolecules to oxidative stress. Since red oranges of Sicily are a major source of phenolic molecules, both integrated and organic species may be an important source of antioxidant phytochemicals in populations with habitually low intakes of antioxidant micronutrients.

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