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Antiradical Activity of Water Soluble Components in Common Diet Vegetables

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The antiradical activity of water-soluble components contained in mushrooms (*Psalliota campestris*), onions (*Allium cepa*), white cabbage (*Brassica oleracea* var. *alba*), and yellow bell peppers (*Capsicum annuum*) against hydroxyl radicals was tested in a chemical and biological system. The vegetable juices were obtained by centrifugation of a vegetable homogenate processed at 2 °C or heated at 102 °C. The chemical system consisted of a buffered reaction mixture composed of Fe(III)-EDTA, 2-deoxy-D-ribose, ascorbic acid, and H_2O_2 generating the hydroxyl radical. The antiradical activity was expressed as an inhibition of deoxyribose degradation. The biological system consisted of IMR32 neuroblastoma cells exposed to H_2O_2 in the presence or absence of the vegetable juices. Cells were pretreated for either 24 h or 1 h with the vegetable juices, and reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used as a cell viability assay. All vegetable juices inhibited the degradation of deoxyribose and increased the viability of H_2O_2 treated cells. Raw mushroom juice proved to be the most active in both cases. Boiling significantly affected the activity of mushroom juice, but did not change significantly the effect on onions and yellow bell peppers, and partially increased the activity of white cabbage juice. Mushroom antiradical activity was also confirmed by a cytofluorimetric analysis.

KEYWORDS: Diet vegetables; hydrogen peroxide; cell death; antioxidant activity

INTRODUCTION

It is common knowledge that the increase in oxidative stress, which particularly occurrs with aging, may be one of the contributing factors in the neuronal death occurring after any ischemic/hypoxic insult, and also in neuro-degenerative disorders such as Alzheimer's and Parkinson's diseases (*1*). For example, the age-related increase in cellular oxidative stress and impairment of energy metabolism results in the disruption of neuronal calcium homeostasis, together with the increased vulnerability of neurons to excitotoxicity and apoptosis (*2*). In addition, oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, including cancer, cardiovascular diseases, and inflammation.

Oxidative stress in cells can result from either an increase in the levels of reactive oxygen species (*3*, *4*) or a reduction of the natural cell antioxidant capacities (*5*). Among free radicals, the hydroxyl radical is one of the most aggressive found in living beings, reacting at a controlled diffusion rate with molecules such as DNA, lipids, proteins, and carbohydrates (*6*). In humans, the antioxidant protection against toxic intermediates is heavily influenced by nutrition (*7*), and it is noteworthy that great interest has arisen about the possibility that antioxidants of plant origin, particularly those derived from common diet vegetables, may reduce the risk of chronic conditions such as cancer and cardiovascular disease, as well as age-related degenerative brain disorders (*8*).

The presence of antioxidant compounds in fruits and vegetables is now considered of great nutritional importance because they are believed to inhibit, either in chemical systems or in vivo, the action of free radicals, and therefore prevent their deleterious effects (*9*, *10*). In recent years, many new antioxidants have been sought within natural sources, especially from edible or medicinal plants (*11*−*13*). Several experimental models are used to demonstrate the antioxidant activities of vegetable extracts. Among others, artichoke extracts have been shown to be protective against hydroperoxide-induced oxidative stress in cultured rat hepatocytes (*14*). We have previously shown that extracts derived from vegetables important in the Mediterranean diet are antioxidant in vitro against lipid peroxidation in the linoleic acid−β-carotene micellar model system (*15*), and protective ex vivo against rat liver microsome lipid peroxidation, induced by CCl_4 (*16*). Moreover, and particularly related to the Mediterranean diet, there are studies involving components of

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olive oil, such as hydroxytyrosol which has been shown to be highly protective against the peroxynitrite-dependent DNA damage in vitro (*17*). Many methods are currently used to attain the successful screening of plant extracts, possibly leading to new therapeutic fields which could be further explored (*18*).

The aim of our work was to verify, in both a chemical and a biological system, the specific antiradical activity of the water-soluble components contained in four common edible vegetables (mushrooms, onions, white cabbage, and yellow bell peppers) widely consumed in the Mediterranean diet, against the hydroxyl radicals, and to then correlate any accordance of the results obtained in the two different systems.

MATERIALS AND METHODS

Vegetable Samples. The vegetables were purchased from a local supermarket and were mushrooms (*Psalliota campestris*), onions (*Allium cepa* L.), white cabbage (*Brassica oleracea* L. var. *alba*), and yellow bell peppers (*Capsicum annuum* L.).

Reagents. Folin−Ciocalteu reagent, chlorogenic acid, sodium bicarbonate, ferric chloride hexahydrate ($FeCl_3$), ethylenediaminetetraacetic acid (EDTA), 2-deoxy-D-ribose, hydrogen peroxide (H_2O_2), ascorbic acid, thiobarbituric acid, trichloroacetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox C), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich S.r.l. (Italy). Fetal bovine serum, penicillin, streptomycin, and glutamine were purchased from Gibco (Paisley, UK), and AnnexinV-fluorescein isothiocyanate (Ann. V) and propidium iodide (PI) were purchased from Bender MedSystem.

Sample Preparation. The vegetables were washed (onions were also peeled), weighed, cut into small pieces, homogenized, and then centrifuged at room temperature for 4 min to completely separate the juice from each vegetable. The volume of each vegetable juice was measured. The juice was filtered in an ice bath (2 °C) first through filter n. 1573, diameter 190 mm (Ruudfilter Schleicher Schuell), and then through membranes of cellulose acetate/cellulose nitrate mixed esters (0.22 μm) (Millipore). All these operations were carried out under a dim light, resulting in loss of most of the juice color. The filtered juice was subdivided into three batches. The first batch was immediately analyzed, and the second batch (10 mL) was boiled for 30 min, which was considered to be the time commonly used in the home cooking of most vegetables. The temperature during boiling was experimentally measured, and the result was 102 ± 0.5 °C. The volume of the boiled juice was brought to the initial volume (10 mL) of the raw juice. The pH value of each sample was immediately measured. The third batch was freeze-dried and its dry weight was determined.

Determination of Total Phenolics. The phenol content in the juices obtained at 2 °C was measured by the Folin−Ciocalteu reagent (*19*) using chlorogenic acid as the standard. A 300 μL aliquot of vegetable juice was added to a conical flask (25 mL) along with 10 mL of distilled water, 1.25 mL of Folin−Ciocalteu reagent, 3.75 mL of sodium bicarbonate (200 g L^{-1}), and the mixture was diluted with distilled water to 25 mL. Samples were well mixed and incubated in the dark for 2 h. Sample absorbance was read with a spectrophotometer (Beckman DU 7500) at 725 nm. Results were expressed as mg of chlorogenic acid in 100 g of fresh edible vegetable.

Deoxyribose Assay. The scavenger activity of the vegetable juices based on the inhibition of the degradation of deoxyribose caused by the attack of hydroxyl radicals was evaluated using the method of Aruoma et al. (*20*) with some modifications.

In a final volume of 1.2 mL, the reaction mixture contained the following reagents at the final concentrations: $FeCl_3$ (25 μM) premixed with EDTA (100 μM) in KH_2PO_4/KOH buffer (pH 7.4), 2-deoxy-D-ribose (2.8 mM), H_2O_2 (2.8 mM), ascorbic acid (100 μM), and 12 μL of vegetable juice (sample), or the same volumes of KH_2PO_4/KOH buffer (control sample). Both samples were placed in a water bath at 37 °C for 1 h, and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.8% trichloroacetic acid were added. The reaction mixtures were heated in a water bath at 80 °C for 20 min, kept in ice for 5 min, and then centrifuged for 5 min at 3000 rpm to separate the particles. The absorbance of the supernatant was read in a spectrophotometer at 532 nm against a solution prepared as described but without ascorbic acid (blank) to correct for interference due to the juice color and thiobarbituric acid-reactive substances that might naturally occur in vegetable juices.

The scavenger activity was expressed as the percentage inhibitory activity (IA%) of degradation of deoxyribose in the presence of the vegetable juice, relative to that of the control sample (without the vegetable juice) using the following equation:

$$\text{IA\%} = 100 - \frac{\text{absorbance sample} \cdot 100}{\text{absorbance control sample}}$$

The scavenger activity was determined also for an aqueous solution of Trolox C that was assayed at three final concentrations of 50, 100, and 250 μM.

Cell Cultures. IMR32 cell cultures (human neuroblastoma) were grown in a RPMI I1640 medium containing 10% (v/v) fetal bovine serum, penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), and 2 mM glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Treatment and Assessment of Cell Viability (MTT Colorimetric Assay) (*21*). At day 0, IMR32 cells were plated at a density of 5×10^4 viable cells per well in 96-well plates coated with 0.5% gelatine. On the day of the experiment, cells were incubated for either 24 h or 1 h at 37 °C and in the presence of the different diluted vegetable juices (1:1000). After this incubation period, cells were treated for 24 h with H_2O_2 at different concentrations. The protective effect of vegetable extract was then evaluated using the MTT colorimetric assay. MTT is an indicator of the mitochondrial activity in living cells. In the present study, cells were exposed to an MTT solution in a phosphate buffer solution at pH 7.4 (1 mg mL^{-1}). Following 3 h incubation with MTT and treatment with sodium dodecyl sulfate for 24 h, the reduction of living cells was quantified using a microplate reader (Bio-Rad model 550).

The protective activity was expressed as the rescue percentage (% rescue) of IMR32 cells in the presence of the vegetable juice, relative to that of the control sample (without the vegetable juice) using the following equation:

$$\text{\% rescue} = \frac{\text{absorbance sample} \cdot 100}{\text{absorbance control sample}} - 100$$

The protective activity was determined also for a 250 μM Trolox C solution.

Cytofluorometric Analysis (*22*). On the day of the experiment, IMR32 cells were plated at a density of 4×10^6 viable cells per flask (25 cm^2) and then incubated at 37 °C for 1 h in the presence of mushroom juice (1/1000). After this incubation period, cells were treated for 4 h with 500 μM H_2O_2. Finally, cells were transferred to a graduated measuring tube, treated with Ann. V and PI, according to kit instructions, and subjected to fluorescence-assisted cell sorting (FACS) analysis, by a cytofluorometer Facstar (Becton & Dickinson, San Jose, CA), with a 200 mW argon laser at 480 nm, used with a gating filter at 610 nm. Ann. V is a binding protein capable of interacting with phosphatidilserine residues that have been transferred from the internal to the external phospholipidic membrane sheet during the apoptotic process. Ann. V is able to characterize apoptotic cells discriminating early and later events. PI enters cells through damaged membranes and binds DNA, evidencing apoptotic and necrotic cells.

Statistical Analysis. The values represent mean values of at least 5 replications for the deoxyribose assay and 3−4 independent experiments on quadruplicate samples for the MTT assay. Data were analyzed using the analysis of variance test, followed by, when significant, an appropriate post hoc comparison such as the Student's *t* test or Dunnet's *t* test: a p value <0.05 was considered significant.

RESULTS AND DISCUSSION

The aim of our investigation was to determine, both in vitro and in a cellular system, the potential antioxidant activity (AA)

Table 1. Summary Description of the Tested Vegetables and Their Juices[a]

name		mL g^{-1}	dried residue mg mL^{-1}	total phenols mg 100 g^{-1}	pH
Psalliota campestris	(mushrooms)	0.34 ± 0.05	53.80 ± 6.16	31 ± 2.5	6.81 ± 0.11[b] 6.47 ± 0.12[c]
Allium cepa	(onions)	0.54 ± 0.04	100.48 ± 18.86	35 ± 2.6	5.61 ± 0.07[b] 5.23 ± 0.13[c]
Brassica oleracea convar. capitata var. *alba*	(white cabbage)	0.47 ± 0.11	74.75 ± 19.43	37 ± 2.3	6.50 ± 0.11[b] 5.91 ± 0.29[c]
Capsicum annuum	(yellow bell peppers)	0.60 ± 0.09	76.24 ± 7.25	118 ± 9.4	5.09 ± 0.11[b] 4.70 ± 0.39[c]

[a] Values represent mean values of six replications. [b] Raw juice. [c] Boiled juice.

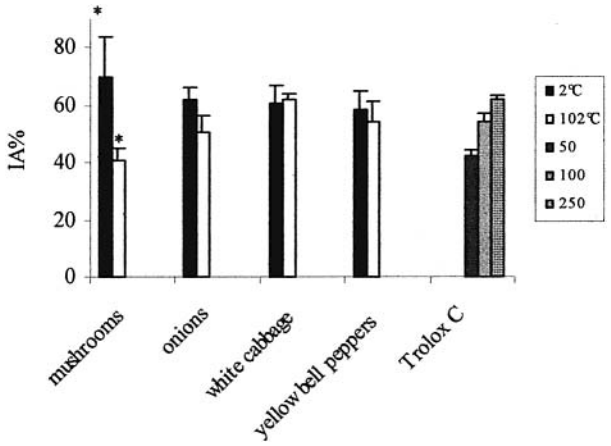

Figure 1. Inhibitory activity percentage (IA%) of raw and boiled vegetable juices and of 50, 100, and 250 μM Trolox C solutions against hydroxyl radical in the deoxyribose assay. The asterisk shows statistical differences.

of the selected water soluble components of four vegetables commonly used in the Mediterranean diet. The AA was determined for the vegetable juices "in toto" obtained by simple centrifugation and filtration at 2 °C in order to minimize the effect of handling. It is well-known that a number of factors (i.e., the antioxidant concentrations, temperature and pH of the medium, the presence of other chemicals with either positive or negative synergism) can strongly influence the antioxidant property (*23*). To verify the effects of thermal treatment to which most vegetables are subjected in home cooking, juices were also analyzed after being boiled at 102 °C for 30 min.

Table 1 summarizes the characteristics of the vegetable juices considered, including the raw juice volume obtained from 1 g of each fresh vegetable, the dry residue, and the total phenol content of the vegetable juices. The pH values of the juices treated at 2 and 102 °C are also reported. The results showed that yellow bell peppers had the higher water and total phenol contents. Onions had the highest water-soluble component content. Mushrooms had the lowest water, total phenol, and water-soluble component contents. Overall, thermal treatment caused a decrease in the pH of the juice from all the tested vegetables.

To test the antiradical activity of the vegetable juices in a chemical system, we used the deoxyribose assay (*20*). The antiradical activity is expressed as the vegetable juice inhibitory activity against the degradation of deoxyribose which acts as the target for the hydroxyl radical attack. Ascorbic acid was used as a reducing agent of Fe(III) to start the reaction.

The percentages of inhibitory activity (IA%) obtained by the chemical assay are reported as a histogram in **Figure 1**. All of the four vegetable juices were shown to possess very high hydroxyl radical scavenger activity. Onion, white cabbage, and yellow bell pepper juices inhibited the degradation of deoxyribose, caused by the attack of the hydroxyl radical, by approximately 60%, and the mushroom juice IA% reached a value as high as 70%. These values are similar to, or higher than, those obtained with a 250 μM solution of Trolox C, a water-soluble analogue of vitamin E. The activity of each vegetable juice was quite constant as shown by the relative standard deviation value (SD).

Regarding the effects of thermal treatment, the results (**Figure 1**) concerning the boiled juices show that there was no effect for white cabbage IA% values, a low decrease for onion and yellow bell pepper values, and a significant change for mushroom juice. The mushroom IA% decreased from 70 to 41%. Nevertheless, boiled mushroom juice maintains an activity similar to that of a 50 μM solution of Trolox C. Differences among the IA% of raw and boiled juices were found to be statistically significant ($p < 0.05$) for mushrooms only. Thermal treatment for mushroom and white cabbage juices seems to stabilize their IA%, as shown by the lower SD values found for boiled juices.

Our second goal was to determine the activity of vegetable juices against H_2O_2-induced cell death. Various cellular models have been used to determine the potential protective activity of vegetable juices against oxidative stress-induced cell death, including rat hepatocytes (*14*), rat cerebella granule cells (*24*), and human neuroblastoma SH-SY5Y (*25*). The cellular model chosen was the neuroblastoma cell line (IMR32), which is sensitive to oxidative stress-induced cell death. A 24 h exposure to 32, 125 and 500 μM H_2O_2 resulted in a concentration-dependent decrease in the percentage of cell viability, by 91.2%, 60.1%, and 12.7%, respectively.

To test the protective activity of these vegetable samples, IMR32 cell cultures were pretreated for either 24 h or 1 h with the vegetable juices before treatment with 500 μM H_2O_2. First of all, we tested the possibility that the vegetable juices might include components with potential cytotoxic activity. However, no significant toxicity of each single juice was detected even at a dilution of 1/100 (data not shown). The vegetable juices were tested diluted 1/1000 in the culture medium.

The MTT assay showed that the viability of IMR32 cells exposed to 500 μM H_2O_2 was increased when cell cultures were preincubated for 24 h with the considered raw vegetable juices (**Figure 2**). In the case of mushroom, rescue percentage (% rescue) was increased by 126.5%, a value similar to that obtained with a 250 μM Trolox C solution (141.6%). In all cases, except for the yellow bell pepper juice (high DS), the increase in % rescue was statistically significant relative to that of the control sample.

For a 1 h preincubation period with raw juices, the cell viability was increased in respect to the 24 h preincubation period, in the presence of the fresh vegetable samples for

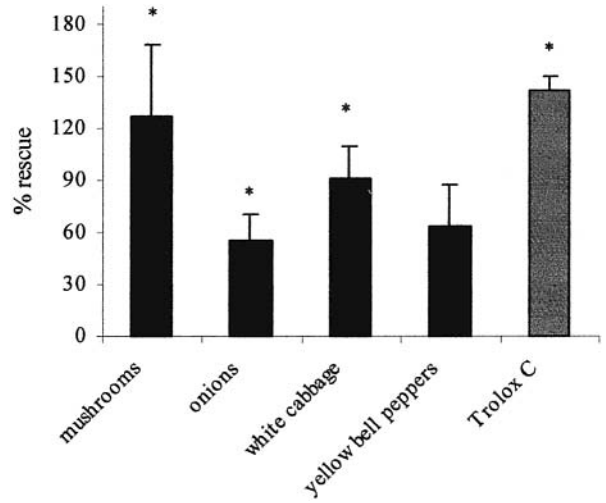

Figure 2. Rescue percentage (% rescue) of IMR32 cells preincubated for 24 h with raw vegetable juices or with a 250 μM Trolox C solution, and then exposed to 500 μM H_2O_2. The asterisk shows statistical differences.

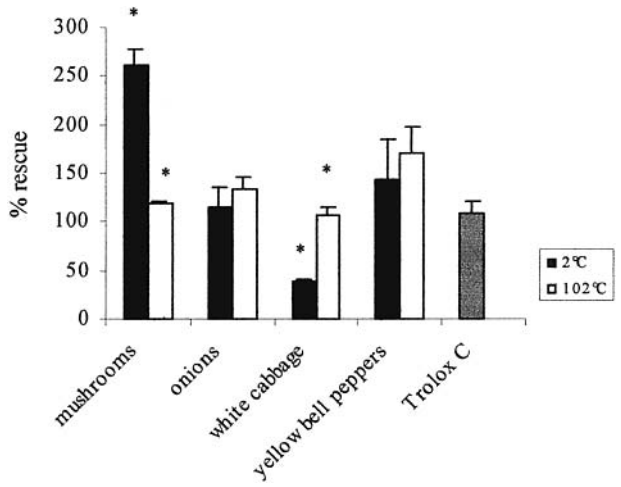

Figure 3. Rescue percentage (% rescue) of IMR32 cells preincubated for 1 h with raw and boiled vegetable juices or with a 250 μM Trolox C solution, and then exposed to 500 μM H_2O_2. The asterisk shows statistical differences.

mushrooms, yellow bell peppers, and onions, but instead decreased for white cabbage (**Figure 3**). In fact, the % rescue for raw mushroom juice reached a value of 260.8%. Similar, but less potent, effects were observed when IMR32 cells were incubated with yellow bell pepper and onion juices, which gave rescue values of 144.3% and 114.8%, respectively. Finally, an exposure to white cabbage for 1 h resulted in a lower increase in MTT values (41.4%). In this case, a 250 μM Trolox C solution gave a value of 109.1%. The increased effectiveness against oxidative stress-induced cell death for a shorter period of exposure to the raw juices compared to that with a pretreatment scheme of 24 h is probably due to the protective components' inherent instability, or perhaps the possibility they could be metabolized and inactivated by the cells over a longer period. The opposite effect was obtained with white cabbage, suggesting the presence of antioxidant components which are more stable or which required a longer time for the integration within the microenvironment.

We also investigated the effects of these vegetable samples, after they were boiled, on IMR32 cell cultures (**Figure 3**). The results showed that thermal treatment of the juices increased the viability of IMR32 cells in comparison to that of the crude juices, with the exception of mushrooms which, previously treated at 102 °C, increased the viability of IMR32 cells exposed to H_2O_2 with reduced efficacy (118.5%) compared to that of the raw juice (260.8%). The boiling of yellow bell pepper juice resulted in an increase in % rescue up to 171.4%; boiled onions gave a value of 132.6%, and the result for white cabbage was 106.1%.

Although the different applied systems can make appropriate comparisons difficult, we note that all tested vegetables showed antiradical activity, with mushrooms being the most active. The behavior of vegetable juices upon thermal treatment somewhat resembles that described in the in vitro antioxidant assays. In particular, the great sensitivity of the mushroom juice to thermal treatment in both experimental models suggests the presence of thermolabile components as the major fraction with anti-hydroxyl radical activity. Concerning the other vegetables, we observed somewhat different behavior, with onions and white cabbage being relatively insensitive to thermal treatment. A major difference between the in vitro activity and the activity on IMR32 cells was noted for the yellow bell pepper juice, suggesting perhaps the involvement of separate components in the antioxidant activity within the two sets of experiments. It has been reported, in fact, that vegetable juices often show the coexistence of oxidant and antioxidant components (*15*, *26*) and, in most cases, the pro-oxidant fraction appears to be thermolabile.

No correlation was found between the IA% and other juice characteristics reported in **Table 1**, including the content of total phenol compounds ubiquitously present in plant materials, to which the protective effects of dietary vegetables are generally attributed. Again, no correlation was found between the above-mentioned characteristics and the effect on cell viability of the juices. In fact, the higher activity shown by the mushrooms in both systems is paralleled by the lowest total phenol content. These findings seem to indicate that other water-soluble components, different from polyphenols and their derivatives, may possess antioxidant activity.

Having established that mushroom juice has the highest protective action, we then sought to characterize the effect of the vegetable on the pattern of cell death induced by H_2O_2 exposure in IMR32 cells. Oxidative stress-induced cell death is a process for which the molecular details have not been fully elucidated. Oxidative stress has been reported to increase intracellular calcium (*27*), but can occur solely in response to free radical overproduction (*28*) and can activate both the processes of apoptosis (*29*) and necrosis (*30*). We have investigated the effect of mushroom juice on H_2O_2-induced cell death by cytofluorimetric analysis, in accordance to the Annexin V/propidium iodide staining method, of the fraction of viable, apoptotic, and necrotic cells. We observed that a 4 h exposure to H_2O_2 (500 μM) induced a significant increase in apoptotic and necrotic cell fractions (**Figure 4a**,**b**,**c**). The effects of H_2O_2 were strongly inhibited by treatment with mushrooms (**Figure 4d**,**e**,**f**). Whereas only a 27.7% cell survival was measured in IMR32 exposed to H_2O_2 action only (**Figure 4c**), a preexposure to mushroom juice, followed by the exposure to H_2O_2, resulted in an increase in cell viability up to 38.4% (**Figure 4f**). These data suggested that mushroom juice could slow the path of cell death induced by H_2O_2.

The present study indicates that the water-soluble components of onions, yellow bell peppers, white cabbage, and, above all, mushrooms demonstrate significant antioxidant activity. At least some of such components can probably react easier than cell

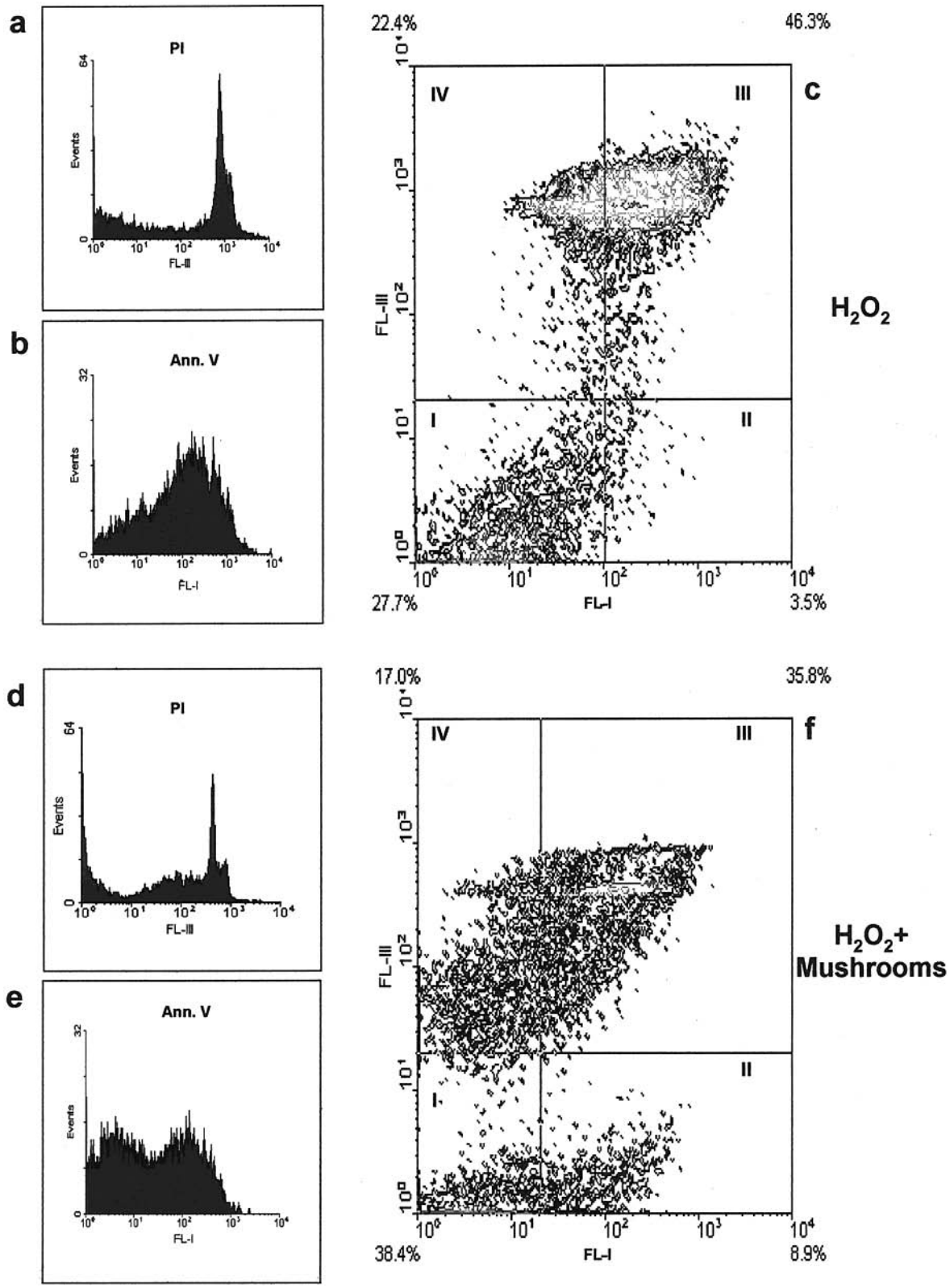

Figure 4. Cytofluorometric analysis of the effect of mushroom juice on H_2O_2-induced cell death. Panels a, b, d, and e are histograms reporting the fluorescence due to PI (FL-III) and to Ann. V (FL-I) vs the number of reacting cells (events). Panels c and f are biparametric diagrams reporting the FL-I vs FL-III. The first quadrant (I) shows a lower density of viable cells (low PI and Ann. V levels) in c than in f. The second quadrant (II) represents early apoptotic cells that have low PI and high Ann.V levels. The third (III) and fourth (IV) quadrants of panel c show higher density of late apoptotic cells (high PI and Ann. V levels) and necrotic cells (high PI and low Ann. V levels) than panel f, respectively.

components with hydroxyl radical. Therefore, they are able to reduce a hydroxyl radical attack on biomolecules such as deoxyribose and to protect and rescue IMR32 cell cultures against H_2O_2-induced toxicity. This is consistent with other results obtained in vitro, in the micellar system linoleic acid−β-carotene (*15*), in a model system of oxidative-stress induced toxicity (*31*), and ex vivo in the rat liver microsomial system (*16*). These findings further support the possibility that these

vegetables contribute to the protective effects on human health of vegetables used in the Mediterranean diet. The specific water-soluble active components of these juices require further research. The considered juices have been proved to be an extremely complex mixture (*32*, *33*) and their various components may have synergistic effects with complex interactions that are still largely unexplored.

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