Antioxidant Polyphenolic Constituents of *Vitis × Labruscana* cv. ‘Isabella’ Leaves

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Abstract: The polyphenolic composition and antioxidant properties of the methanol leaf extract of *Vitis × labruscana* cv. ‘Isabella’ has been performed by means of different spectrophotometric antioxidant methods. Intracellular ROS production was measured by cell fluorescence after loading with 2',7'-H2DCF-DA. The extract promising efficacy has laid the basis to its phytochemical characterization. Three hydroxycinnamoyl tartaric acids (1-3), isoquercitrin 4, and five flavonol glycuronides (5-9) were isolated from *Vitis × labruscana* cv. ‘Isabella’ leaves. Chemical structures were elucidated on the basis of their spectroscopic features. Metabolites 6, 7, and 9, characterized by glucuronic or galacturonic acid methyl ester, are described from the genus *Vitis* for the first time. The metabolites isolated as major constituents were investigated for their DPPH radical scavenging capacity.

Keywords: *Vitis × labruscana* cv. ‘Isabella’, polyphenols, flavonoids, antioxidant activity.

1. INTRODUCTION

The different tissue and cellular components of plants are a rich source of antioxidant molecules. The term antioxidant refers to those substances able to defend organisms against free radicals, by inhibiting their formation or counteracting their attack on biological targets. In fact, antioxidants are able to supply electrons to free radicals thereby restoring the chemical balance of the systems in which these reactive species act [1].

The physiological and continuous biosynthesis of free radicals can massively increase as the result of an imbalance between the intra- and extracellular environment. This condition, best known as oxidative stress, is considered one of the main factors, which trigger various degenerative chronic diseases. In order to reduce or eliminate the excess of reactive species and its harmful activity, exogenous antioxidants should be introduced to assist the activity of the endogenous antioxidant system. The fame of beneficial factors attributed to a large number of phytochemicals, such as polyphenols, α-tocopherol and ascorbic acid, springs from their effective radical scavenging action that, in vivo, seems to be closely related to vital biological functions such as anti-mutagenicity, anti-carcinogenicity and anti-aging [2]. Indeed, the significant investment of resources in the search for new antioxidant phytochemicals is justified by several areas in which these substances are used or can be used.

Today more than ever, the antioxidant natural products are employed as (1) lead compounds in studies aimed at the development of anticancer drugs with potentially greater specificity and fewer toxic side effects, (2) active ingredients to be used in prevention strategies vs. chronic degenerative diseases, (3) additives for foods and beverages in place of synthetic preservatives and (4) templates in the production of food supplements and functional food.

As part of research aimed at identifying new molecules with antioxidant properties of edible plant species [3-6] we carried out the phytochemical analysis of *Vitis × labruscana* cv. ‘Isabella’ leaves.

*Vitis × labruscana*, known in Italy as ‘fragola’ (strawberry) grape, is a subgroup of grapes originating from a hybridization of *Vitis labrusca* and other *Vitis* species. The cultivar ‘Isabella’, obtained by a cross with an unknown *Vitis vinifera* susceptible to mildew and black rot, was imported into Europe in the 19th century because of its high resistance to grape phylloxera [7]. To the best of our knowledge, no phytochemical studies have been carried out on this hybrid. Shure and Acree reported the partial characterization of β-damascenone precursors in cell cultures of *Vitis × labruscana* cv. ‘Concord’ grapes [8]. Recently an HPLC-DAD-ESI-MS study of anthocyanins, pyrananthocyanins, flavonols and hydroxycinnamic acid derivatives present in ‘Isabella’ wines was performed in order to evidence differences between these and *Vitis vinifera* red wines [9]. The total phenol content and the antioxidant activity of ‘Isabella’ grape has been also reported [Soares et al., 2008] [10].

Previously, an extensive antioxidant screening of the different components of the species (stalk, leaf, stem, seed, peel and pulp) was carried out [11]. The leaf extract promising
activity and the awareness of the effectiveness of *Vitis vinifera* leaves, already widely used as active ingredient in the pharmaceutical and cosmetics, have directed our study to a further evaluation of the extract antioxidant activity on cell systems and to the purification, by means of chromatographic techniques, and spectroscopic identification (especially by 1D and 2D NMR) of its main phytochemicals. Three hydroxycinnamoyl tartaric acids (1-3), isoquercitrin 4, and five flavonol glycosides (5-9) have been isolated. The metabolites 1, 4, 5, 7 and 8, isolated as major constituents of leaf methanol extract, have been screened for their DPPH radical scavenging capacity.

2. MATERIALS AND METHODOLOGIES

2.1. Plant Material and Sampling

Leaves of *Vitis × labruscana* cv. ‘Isabella’ (476.0 g) were collected in Garzano, near Caserta (Southern Italy), in the autumn 2008. A voucher specimen has been deposited at the Herbarium of the Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche of the Second University of Naples.

In order to assess leaf antioxidant efficacy a rate of 76.0 g was suitably reduced to fragments and lyophilized by Flexi-Dry MP (FTS Systems, Vienna, Austria). The obtained freeze-dried powders were extracted by sonication (Dr. Hielcher UP 200S, Berlin, Germany) at maximum power for 2 h using pure methanol as extracting solvent, and subsequently centrifuged at 3500 rpm for 10 min in a Beckman GS-15R centrifuge (Beckman Coulter, Milano, Italy) fitted with rotor S4180. After removal of the solvent, we obtained crude leaf methanol extract, have been screened for their DPPH scavenging capacity.

The remaining sample (400 g) was macerated in methanol for 3 days at 4 °C. The obtained crude extract (36.5 g) was stored at −20 °C until further analyses.

2.2. General Experimental Procedures

NMR spectra were recorded on a Varian 300 spectrometer Fourier transform NMR in CD3OD at 25 °C. UV spectra were performed on UV-1700 Shimadzu spectrophotometer in MeOH solution. Analytical TLC was performed on Merck Kieselgel 60 F254 or RP-8 F254 plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H2SO4/AcOH/H2O (1:20:4). The plates were then heated for 5 min at 120 °C. Preparative TLC was performed on Merck Kieselgel 60 F254 plates, with 0.5 or 1.0 mm film thickness. Column chromatography (CC) was performed on Merck Kieselgel 60 (63-200μm), Baker RP-8 silica gel (40-63μm), Fluka Polyamide 6 (50-160μm), Flash Column chromatography (FCC) was performed on Merck Kieselgel 60 (40-63μm) in pure N2 pressure.

2.3. Cell Cultures and Determination of Intracellular ROS Level

Human hepatoblastoma cells (HepG2) and lung epithelial cells (A549) were purchased from ICLC (Interlab Cell Line Collection) at Istituto Nazionale per la Ricerca sul Cancro, Genoa (Italy). HepG2 were grown in RPMI containing 10% fetal bovine serum, 50 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO2. A549 cells were plated and grown under the same conditions, except that DMEM high glucose was used instead of RPMI.

In order to define the antioxidant activity of leaf extract from *Vitis × labruscana* cv. ‘Isabella’ two experimental protocols were adopted. Both of these protocols aimed to evaluate the intracellular content of reactive oxygen species (ROS) by 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Sigma) assay. A549 and HepG2 cells were seeded in 96-multiwell black plates (PBI International) at a density of 4 × 104 cells/well. The first experimental adopted provided, after 48 h of incubation, the pre-treatment of cells with a dose of leaf extract from *Vitis × labruscana* cv. ‘Isabella’ able to induce an inhibition of cell viability by about 30% (IC30). After further 24 h of incubation, cells were treated first with 2',7'-H2DCF-DA (5.0 μM) for 1 h at 37 °C and then, after being properly washed with PBS (100.0 μL x 3), exposed for 40 min to pro-oxidant H2O2 (500.0 μM). In the second experimental protocol after 48 h of incubation, cells were treated with 2',7'-H2DCF-DA (5.0 μM) for 1 h at 37 °C and then, after being properly washed with PBS (100.0 μL x 3), simultaneously exposed for 40 min to the investigated extract and pro-oxidant H2O2 (200.0 μM).

2',7'-H2DCF-DA is a permeable and non-fluorescent molecule that diffuses passively into the cells where it is hydrolyzed by intracellular esterases to form 2',7'-H2DCF. The presence of ROS species in the intracellular environment causes oxidation of 2',7'-H2DCF to 2',7'-dichlorodihydrofluorescein (DCF), a highly fluorescent molecule. The fluorescence emitted is proportional to the intracellular ROS content. It was monitored using an excitation wavelength of 485 nm and an emission wavelength of 538 nm in a TECAN fluorimeter LS 55 (Luminescence Spectrometer, PerkinElmer Ltd.). The results were expressed as percentage of intracellular ROS level of the cells treated with the extract compared to an untreated control.

2.4. Fractionation and Isolation

Leaf methanol crude extract was dissolved in water and shaken with EtOAc. The aqueous fraction (23.0 g) was chromatographed on Amberlite XAD-4 and eluted first with water and then with methanol. The methanol eluate was chromatographed on SiO2 FCC, eluting with [CHCl3/MeOH/H2O (13:7:3, lower phase), furnished pure metabolites 6 (17 mg), 9 (4 mg); the second fraction, purified by TLC (0.5 mm) with [CHCl3/MeOH/H2O (13:7:3, lower phase), furnished pure metabolites 4 (17 mg), 7 (2 mg) and 8 (3 mg). Fraction B was chromatographed by RP-8 CC eluting with [MeCN/MeOH/H2O (1:1:3) was purified by TLC (1.0 mm) using [EtOAc/MeOH/H2O/HCOOH (16:2:1:1) as eluent, yielding fractions 5 (27 mg). Fraction C was chromatographed by CC on Polyamide 6 resin obtaining a fraction, whose NMR spectra evidenced the presence of cinnamoyltartaric acids. This fraction was chromatographed by SiO2 FCC, eluting with
1H-NMR (300 MHz, CD3OD) δ: 3.00-3.60 (ov, H-2"-H-5"), 5.21 (d, J 7.6 Hz, H-1"), 6.17 (d, J 2.0 Hz, H-6), 6.35 (d, J 2.0 Hz, H-8), 6.88 (d, J 8.3 Hz, H-5'), 7.59 (dd, J 8.3 e 2.2 Hz, H-6'), 7.61 (d, J 2.2 Hz, H-2'), 104.4 (C-6), 104.1 (C-1"), 116.0 (C-2"), 117.5 (C-5"), 123.2 (C-6"), 135.6 (C-3"), 145.9 (C-4"), 149.9 (C-3'), 158.6 (C-2'), 159.1 (C-9), 162.9 (C-5'), 167.4 (C-7'), 179.3 (C-4').

2.4.1.5. Quercetin 3-O-β-D-glucopyranoside (5)

UV (MeOH) λmax nm 363, 261; 1H-NMR (300 MHz, CD3OD) δ: 3.00-3.60 (ov, H-2"-H-5"), 5.30 (d, J 7.2 Hz, H-1"), 6.19 (d, J 1.2 Hz, H-6), 6.38 (d, J 1.2 Hz, H-8), 6.87 (d, J 8.1 Hz, H-5'), 7.49 (dd, J 8.1 e 1.5 Hz, H-6'), 7.94 (d, J 1.5 Hz, H-2'), 104.5 (C-10), 116.2 (C-2'), 118.1 (C-5'), 122.8 (C-6'), 135.8 (C-3), 145.9 (C-4'), 149.9 (C-3'), 158.6 (C-2'), 159.1 (C-9), 162.5 (C-5), 166.7 (C-7), 176.4 (C-6"'), 179.4 (C-4').

2.4.1.6. Quercetin 3-O-β-D-glucuronide-6"-methyl ester (6)

UV (MeOH) λmax nm 363, 261; 1H-NMR (300 MHz, CD3OD) δ: 3.00-3.60 (ov, H-2"-H-5"), 5.30 (d, J 7.2 Hz, H-1"), 6.17 (d, J 1.2 Hz, H-6), 6.36 (d, J 1.2 Hz, H-8), 6.88 (d, J 8.3 Hz, H-5"), 7.43 (d, J 8.3 e 2.1 Hz, H-6"), 7.91 (d, J 2.1 Hz, H-2"). 13C-NMR (75 MHz, CD3OD) δ: 52.2 (OCH3), 73.1 (C-5"), 74.9 (C-4"), 77.3 (C-3"), 77.9 (C-2"), 95.1 (C-8), 100.2 (C-6"), 101.5 (C-1"), 104.5 (C-10), 116.1 (C-2"), 118.5 (C-5"), 135.8 (C-3), 145.9 (C-4"), 149.9 (C-3'), 158.6 (C-2'), 159.1 (C-9), 162.5 (C-5), 166.7 (C-7), 173.2 (C-6"), 179.1 (C-4).
3. RESULTS AND DISCUSSION

A previous study showed the potent antioxidant efficacy and the antiproliferative effects of not edible *Vitis × labruscana* cv. ‘Isabella’ components seed, leaf and stalk [11]. Metabolic profiling studies allowed to evidence that the same components contain a high content of phenolic and flavonoid compounds, which could be responsible for the strong radical scavenging and antioxidant properties observed. In order to clear their antioxidant activity in cell systems the measurement of intracellular ROS level was carried out by means of two different experimental protocols. The experiments were performed toward A549 and HepG2 cell lines using only the antiproliferative extracts at a dose equal to their IC_{30} value calculated on the basis of antiproliferative effects detected after 48 h exposure [11]. The results, expressed as a percentage of ROS formation vs. an untreated control, demonstrate the ability of the extracts to induce a change, although different, in the ROS baseline production (Fig. 1). The simultaneous administration of leaf extract and H_{2}O_{2} resulted in a massive protective effect: the extract seems able to deactivate the oxidant species by defining a percentage reduction of intracellular ROS production of 33.3% in HepG2 cells and 30.9% in A549 cells. The effectivness in inducing the decrease in the level of cellular oxidation was more pronounced when the cells underwent 24 h pre-treatment with the antioxidant extract.

The potent antioxidant activity together with the marked antiproliferative efficacy of the leaf extract suggests it be studied phytochemically. Although little is known about the phytochemical composition of leaves of *Vitis × labruscana* cv. ‘Isabella’, extensive knowledge of the medicinal properties of the commonly used grape vine (*Vitis vinifera* L.) can be traced far back in history. In Ayurvedic (Indian) system, grape leaves are used as a folk remedy for the treatment of diarrhea and vomiting. In Europe, the leaves of *Vitis vinifera* are documented in the literature of traditional medicine for their astringent and homeostatic properties where they are utilized in the treatment of diarrhea, bleeding, haemorrhoids, varicose veins and other circulatory diseases.

Thus, *Vitis × labruscana* cv. ‘Isabella’ leaf crude extract was separated by chromatographic techniques into its constituents. Nine pure compounds were characterized by spectroscopic analysis using, in particular, mono- and two-dimensional NMR experiments and by comparison of their UV and 1^H NMR data with literature values.

Structures of isolated phytochemicals are reported in Fig. (2). Compound 1 was identified as trans-caftaric acid (2-O-caffeoyltartaric acid). Its 13C NMR spectrum showed thirteen signals identified, on the basis of a DEPT experiment, as seven methines and six quaternary carbons. 1H NMR spectrum was in accordance with the presence of a trans-caffeoyl moiety. In the downfield region of the spectrum two olefinic protons as an AB spin system at δ 7.55 (J = 15.9 Hz) and δ 6.36 (J = 15.9 Hz) and three protons of a 1,2,4-trisubstituted aromatic ring as a double doublet at δ 6.91 (J = 7.8 Hz) and an ortho-coupled doublet at δ 6.76 (J = 7.8 Hz) were evident. Furthermore the spectrum displayed two doublets at δ 4.51 and 5.40 attributable to two protons geminal to oxygenated functions. The HMBC experiment allowed us to evidence the heterocorrelation between the proton at δ 4.51 (H-3) and the carbons at δ 175.8 (C-4) and 178.0 (C-1) and between the proton at δ 5.40 (H-2) and the carbons at δ 74.0 (C-3), 175.8 (C-4) and 168.9 (C-9') suggesting the presence of a tartaric acid bound to the C-9' carbon of the caffeoyl moiety. Compound 2 was identified as coutaric acid (p-coumaroyltartaric acid). The 1^H NMR spectrum showed
significant differences compared with 1 in the aromatic region. In fact characteristic signals of a AA'BB' system as two doublets at δ 7.07 and 6.93, assignable to H-2'/H-6' and H-3'/H-5' protons, were in accordance with the presence of a 1,4-disubstituated aromatic ring. The presence of coumaroyl moiety, esterified through its C-9' carbon to the C-2 carbon of the tartaric portion, could be inferred from trans olefinic protons at δ 7.55 and 6.43, assignable to H-7' and H-8', whose coupling constant value (J = 16.8 Hz) suggested a trans geometry, and from the two doublets at δ 4.51 and 5.35. Compound 3 was identified as the cis isomer of compound 1. The 1H NMR spectrum displayed in the downfield region the three protons of the 1,2,4-trisubstitued aromatic ring at δ 7.47 and 5.96 and the two doublets at δ 7.07 and 6.93, assignable to H-2'/H-6' and H-3'/H-5' protons, were in accordance with the presence of a 1,4-disubstituted aromatic ring. The presence of coumaroyl moiety, esterified through its C-9' carbon to the C-2 carbon of the tartaric portion, could be inferred from trans olefinic protons at δ 7.55 and 6.43, assignable to H-7' and H-8', whose coupling constant value (J = 16.8 Hz) suggested a trans geometry, and from the two doublets at δ 4.51 and 5.35. Compound 3 was identified as the cis isomer of compound 1. The 1H NMR spectrum displayed in the downfield region the three protons of the 1,2,4-trisubstitued aromatic ring at δ 7.47 and 5.96 and the two doublets at δ 4.50 and 5.34 of the tartaric moiety. The H-7'/H-8' coupling constant value (J = 2.1 Hz) suggested a cis geometry of the caffeoyl moiety. Hydroxycinnamic acid esters of tartaric acid have been ubiquitously reported as components of fruits and wines of red and white V. vinifera varieties [12]. Recently Liazi et al. standardized a LC method for the determination and quantification of polyphenols in grapes and derived products; they reported caftaric acid (1) and coutaric acid (2) as significant components of many grape varieties [13].

Compound 4 was identified as quercetin-3-O-β-D-glucopyranoside on the basis of NMR data and comparison with pure standard compound. Compound 5 was pure quercetin-3-O-β-D-glucuronide (miquelianin). It showed UV (λ<sub>max</sub> nm 363, 261) and 1H NMR spectra similar to those of the previous compound. The 13C NMR spectrum showed, besides the aglycone characteristic signals, a carbon of a carboxylic function at δ 176.4 and four oxygenated carbons at δ 73.4, 75.6, 77.6 and 78.1 in accordance with the presence of a glucuronic acid as glyconic moiety.

Compound 6 exhibited a spectroscopic pattern similar to that of metabolite 5. The 13C NMR spectrum showed a further signal at δ 52.2 heterocorrelated, in a HSQC experiment, to the singlet, integrated to three protons, at δ 3.67. This latter was heterocorrelated in a long-range experiment to the estereal carbon at δ 173.2. These data allowed us to identify the molecule as the 6'-methyl ester of 5. Metabolite 7 was identified, on the basis of their spectroscopic features, as quercetin-3-O-β-D-galacturonide-6''-methyl ester. The 1H NMR spectrum showed, besides the characteristic signals of quercetin, an anomeric doublet at δ 5.12 and overlapped signals in the range 3.00 – 3.60 ppm. The 13C NMR again underlined the presence of an oxidated glyconic component. In a HSQC experiment, the anomeric proton resulted heterocorrelated to the carbon at δ 105.6.

The 1H NMR spectrum of compound 8 showed some important differences with that of compound 5 in the aromatic region. In fact, besides the signals due to H-5 and H-7 protons, two doublets at δ 8.06 and 6.86 were evident according to the presence of a kaempferol 3-O-β-D-glucuronide. Finally metabolite 9 was kaempferol-3-O-β-D-glucuronide-6''-methyl ester. The spectroscopic pattern was similar to that of metabolite 8 but, as for compound 6, the 13C NMR evidenced a further carbon at δ 51.9 heterocorrelated, in a HSQC experiment, to the singlet at δ 3.65. The heterocorrelation evidenced in an HMBC experiment between the protons at δ 3.65 and the estereal carbon at δ 173.3 allowed us to identify the molecule. The presence of flavonoids has been documented in grape leaves for quite a time. Several hydroxyl flavonoids, some of which never found in the taxon, were isolated from the leaves of some Maroccan Vitis vinifera cultivars [14]. Compounds 4 and 5 are the main components of red wine leaf extract (RWLE), an herbal medicine used in...
the treatment of chronic venous insufficiency and Type 1 diabetes [15].

Compound 8 has been also reported as constituent of several grape varieties [16, 17]. Compounds 6, 7 and 9 are characterized as methyl esters derivatives at the C-6 carbon of glycuronic moiety. Although these latter compounds have been reported from different plant organisms [18, 19], no information is present in the scientific literature on their presence in Vitis species.

The large amount of polyphenols (including phenolic acids, flavonoids, and anthocyanins) in grapes has a key role in the characterization and classification of the wide range of varieties [20] and defines the important beneficial properties of grapes. Several researches highlighted that these compounds are one of the most important quality parameters of grapes since they contribute to grape organoleptic properties and most of them display biological properties of interest, related to their antioxidant efficacies [21].

In order to assess the antioxidant power of metabolites 1, 4, 5, 7 and 8, isolated as major constituents of leaf methanol extract, DPPH method was performed. The antioxidant HAT/ET test estimates the scavenging effect towards the artificial, stable and commercially available DPPH radical; the method, easy to perform and highly reproducible, has been extensively applied on the study of antioxidant activity of wines [22]. The results obtained (Fig. 3) are expressed in terms of DPPH radical scavenging capacity (RSC,%). The metabolite 1 was able to reduce the radical target by 71.9% at the highest tested concentration. The recorded dose-response increase seems due to the presence in the molecule of numerous hydroxyl functions. The metabolite, tartaric ester of caffeic acid, is known in literature as the main constituent of various varieties of Vitis vinifera. The molecule seems to be responsible for important inflammatory and antioxidant properties [23]. The active components are ascribed to the moiety of caffeic acid, polyphenol highly ubiquitous in nature, which is known for its protective effects in the liperoxidation processes, and to tartaric moiety. Molecules 4, 5 and 7, characterized by aglycone quercetin as common component, induced a strong reduction of the DPPH radical. The antiradical capacity is massive for molecules 4 and 5 capable of determining the complete conversion of the radical in its reduced form. The molecules induce a reduction of 79.9 and 73.8% at 30μM concentration. Results emphasize that the presence of different sugar components differently modulates the intensity of the antioxidant response. In fact, the metabolite 7, presenting as glyconic moiety galacturonic acid, C-4 epimer of glucuronic acid, was less active than metabolite defining a reduction of radical species of 62.8% at 100μM concentration. The presence of only one hydroxyl function on B-ring of the flavonol 8 appears to reduce drastically the radical scavenging activities.

Isoquercitrin (4) and its oxidized derivative miquelianin (5) were the most active metabolites. The biological importance of these substances is strongly emphasized by numerous literature data. In particular miquelianin is reported as a flavonol glucuronide constituent of wine and other different species as Nelumbo nucifera [24] and, Hypericum [25] and Salvia species [26]. The molecule, also known as quercitrone, is also a rat plasma quercetin metabolite [27]. It shows an antioxidant effect in human plasma. In vitro studies indicate that miquelianin is able to reach the central nervous system (CNS) from the small intestine [28]. Isoquercitrin (4) and miquelianin showed antidepressant activity in low concentrations in animal models. The efficacy of conjugated quercetin metabolites as attenuators for oxidative stress in CNS was also assessed: miquelianin was effective in suppressing the formation of ROS [29]. Thus, Vitis × labruscana cv. ‘Isabella’ leaf appears a good candidate medicinal plant drug containing inherent active ingredients useful to counteract different degenerative disorders.

4. CONCLUSION

The chromatographic fractionation of the antioxidant methanol leaf extract of Vitis × labruscana cv. ‘Isabella’ led us to the isolation of nine polyphenol metabolites identified on the basis of their spectroscopic features, and by comparison of their UV and 1H NMR data with literature values.

The phytochemicals 1, 4, 5, 7, and 8 were isolated as major constituents and screened for their in vitro antioxidant activity by DPPH assay.

The promising efficacy of some metabolites suggests their possible role as antioxidant agents in order to improve antioxidant status and counteract the risk of diseases associ-
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REFERENCES


