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A selection of grapes from Sardinia: antioxidant properties

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Selezione di uve dalla Sardegna: proprietà antiossidanti

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Estratti di uva, acido linoleico, colesterolo, cellule CaCo-2, antiossidanti

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Summary
Several studies have suggested that grape extracts possess different biological actions and may be beneficial in the prevention of many human diseases, associated with oxidative stress; however rather scarce data on the biological properties of the countless varieties of grapes growing in Sardinia are available. Grape and wine extracts are known to contain high levels of polyphenolic compounds. Polyphenols have been proposed to exert beneficial effects in a multitude of disease states, including cancer, cardiovascular diseases and neurodegenerative disorders. Many of the biological actions of polyphenols have been attributed to their antioxidant properties. In the present study we investigated the antioxidant effect of extracts from 6 varieties of grape growing in Sardinia (Cannonau, Cabernet-sauvignon, Malvasia, Vermentino, Chardonnay, Sauvignon), in simple in vitro systems, during autoxidation of linoleic acid at 37°C and during cholesterol oxidation at 140°C, in the absence of solvent. The cytotoxicity and the capacity of grape extracts to inhibit MDA production were evaluated in the human colon adenocarcinoma cell line, Caco-2. This cell line spontaneously undergoes full differentiation in vitro with enterocyte-like features and has been recognized as a suitable model for evaluating the effect of nutrient components. All tested grape extracts showed no cytotoxicity in the range 25-100 μg/ml and showed a significant antioxidant activity, beeing the native grape extracts from Sardinia the most active.

Riassunto
Numerosi studi hanno messo in evidenza che l'estratto di uva possiede diverse proprietà biologiche che possono essere importanti nella prevenzione delle patologie associate allo stress ossidativo; sono però scarsi i dati sulle proprietà biologiche delle innumerevoli varietà di uve che crescono in Sardegna. Gli estratti di uva e di vino sono noti per contenere elevate concentrazioni di composti polifenolici. Ai polifenoli sono stati ascritti numerosi effetti benefici in differenti stati patologici, tra cui tumori, patologie cardiovascolari e neurodegenerative. Molte delle azioni biologiche dei polifenoli sono state attribuite alle loro proprietà antiossidanti. In questo lavoro abbiamo valutato l'effetto antiossidante degli

estratti di 6 varietà di uva coltivate in Sardegna (Cannonau, Cabernet-sauvignon, Malvasia, Vermentino, Chardonnay, Sauvignon), in semplici sistemi in vitro, durante l'autossidazione dell'acido linoleico a 37°C e durante l'ossidazione del colesterolo a 140°C, in assenza di solvente. La citotossicità e la capacità degli estratti di uva di inibire la produzione di MDA sono state valutate in cellule umane di adenocarcinoma di colon, Caco-2. Questa linea cellulare, in vitro, subisce spontaneamente una completa differenziazione, assumendo le caratteristiche degli enterociti ed è stata riconosciuta come un modello adatto per valutare l'effetto dei componenti nutrizionali. Tutti gli estratti di uva testati non hanno mostrato citotossicità nel range di 25-100 μg/ml e hanno mostrato una significativa attività antiossidante, gli estratti delle uve tipiche della Sardegna hanno mostrato la maggiore attività.

Introduction

Consumption of appropriate amounts of fruits and vegetables is considered essential for the prevention of several diseases. In this respect grapes and other dietary constituents derived from it like grape juice and wine have attracted a great deal of attention in recent years (1). The dietary consumption of grape and its products is associated with a lower incidence of degenerative diseases and certain types of cancers (2).
Grape fruit contains various nutrient elements, such as vitamins, minerals, carbohydrates, edible fibers and phytochemicals. Polyphenols are the most important phytochemicals in grape because they possess many biological activities, such as antioxidant, cardioprotective, anticancer, anti-inflammatory, antiaging and antimicrobical properties (3); in particular, epidemiological studies suggested a protective role of these compounds towards human diseases associated with oxidative stress (4, 5). These data are supported by many in vitro studies, demonstrating the antioxidant, antiproliferative and pro-apoptotic activity of these compounds in many normal and tumor cell lines (1, 2). Grape polyphenols mainly include anthocyanins, flavanols, flavonols, stilbenes and phenolic acids (6, 7). Anthocyanins are pigments, and mainly exist in grape skins, they're the main polyphenolics in red grapes, while flavan-3-ols are more abundant in white varieties (8, 9). Flavonoids are widely distributed in grapes, especially in seeds and stems, and principally contain catechins, epicatechin and procyanidin polymers. Quantitative and qualitative distribution of polyphenols in grape may show significant differences, depending on several factors, such as the varietal differences of Vitis vinifera and the location of cultures (10).
The objectives of this study were to compare the antioxidant capacities of extracts from six Vitis vinifera grape varieties cultivar in Sardinia (three native: Cannonau, Malvasia, Vermentino and three non-native types: Cabernet-sauvignon, Chardonnay and Sauvignon), in simple in vitro systems, during autoxidation of linoleic acid at 37°C and during cholesterol oxidation at 140°C, in the absence of solvent. To determine the possible protective effect of the

extracts against the oxidative damage to intestinal mucosa, we evaluated the inhibition of MDA production in the human colon adenocarcinoma cell line, Caco-2. Caco-2 cells spontaneously undergoes full differentiation in vitro with enterocyte-like features (11) and, since the intestine is the primary site of exposure to substances present in food, this cell line has been recognized as a suitable model for evaluating the effect of nutrient components, for both normal dietary constituents and additives, contaminants, toxicants and drugs (12, 13).

Materials and Methods

All solvents used were HPLC grade (Merck, Darmstadt, Germany); all other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) or CIBA-Geigy (Basel, Switzerland) and were of the highest available purity. The grape extracts ware prepared as methanolic extracts and analysed as previously described (14). The main phenolic compounds quantified are shown in Table 1.

Linoleic acid autoxidation assay.

Linoleic acid autoxidation was conducted in dry state as previously described (15). Samples of 0.5 ml (3566 nmoles) of linoleic acid solution (2 mg/ml in MeOH) were dried down in a glass round-bottom test tube under vacuum. The samples were incubated in a water bath at 37°C for 32 h. Artificial light exposure was kept throughout the experiment. Controls were kept at 0°C. Different amounts of grape extracts (5-100 μg) in MeOH solution (1 mg/ml) were incubated, in dry state, with linoleic acid, before its autoxidation as described above. Analyses of linoleic acid and its oxidation products, the conjugated diene linoleic acid hydroperoxydes (c,t- and t,t-HPODE) were carried out with a HPLC-DAD (15).

Cholesterol oxidation assay.

Cholesterol oxidation was conducted in dry state as previously described by Kim and Nawar (16) with a few modifications. Briefly samples of 0.5 ml (2586 nmoles) of cholesterol solution (2 mg/ml in MeOH) were dried down in a glass round-bottom test tube under vacuum. Samples were incubated in a bath at 140°C for 90 min. Artificial light exposure was kept throughout the experiment. Controls were kept at 0°C. Different amounts of grape extracts (5-100 μg) in MeOH solution (1 mg/ml) were incubated in dry state with cholesterol before its oxidation. Analyses of cholesterol and 7-ketocholesterol were carried out with a HPLC-DAD (17).

Cell culture

Caco-2 cell line was purchased from ECACC (Salisbury, Wiltshire UK). Cell culture media and supplements were purchased from Invitrogen (Milano, Italy). Subcultures of the Caco-2 cells were grown in T-75 culture flasks and passaged with a trypsin-versene solution. Cells were cultured in MEM medium supplemented with 20% FBS, 1% non essential amino acids, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO2. For experimental studies Caco-2 cells, at passage 45-60, were plated at a density of about 1x105/ml and used 21 days post seeding.

Cytotoxic activity

The cytotoxic effect caused by exposure to increasing concentration of grape extracts was assessed on Caco-2 cells, seeded in 24 well plates. Before the treatment old media was removed and then fresh media was added (490 μL). Ten μL of compounds dissolved in MeOH were added to each well (0-100 μg/ml final concentration). After 24 h incubation the cell viability test alamarblue (18) was conducted to determine whether the treatment exerted any toxic effect. After the treatment, the media was discarded and 1 ml of ala-

Tabella 1 -Quantitative analysis of major phenolic compounds in grape extracts

Compunds	mg/kg of freeze-dried grapes					
	Cannonau	Cabernet	Vermentino	Malvasia	Chardonnay	Sauvignon
Hydroxybenzoic acids						
Gallic acid	13,65	24,39	3,46	30,49	22,38	15,93
Hydroxycinnamic acids						
trans-caftaric acid*	30,27	12,27	14,09	21,07	31,81	n.d.
trans-fertaric acid*	15,47	3,80	9,45	15,62	7,69	n.d.
Stilbenes						
t-resveratrol-3-O-glucoside***	0,25	0,24	0,95	0,29	0,46	n.d.
Alcohols/related compounds						
Tyrosol*	11,73	9,47	10,21	16,28	16,08	212,2
Flavanols						
catechin	159,54	456,76	78,03	228,39	486,11	154,03
Procyanidin 1**	284,26	723,38	68,09	564,84	637,51	n.d.
Procyanidin dimer 2**	81,08	106,85	32,19	109,79	1543,35	128,02
Procyanidin trimer 5**	n.d.	n.d.	78,21	305,51	1697,18	n.d.
Flavonols						
Quercetin-3-O-glucoside	190,39	81,11	87,16	377,85	39,17	47,97
Quercetin-3-O-glucoronide***	73,55	51,85	55,60	117,70	27,94	n.d.
Quercetin-3-O-galactoside***	20,02	4,92	10,85	80,47	8,42	1,12
Anthocyanins						
Malvidin-3-O-glucoside	1663,47	6248,34				
Malvidin-3-O-acetylglucoside	157,85	3124,17				

n.d.: not determined
(*) quantified as mg/kg equivalents of gallic acid
(**) quantified as mg/kg equivalents of procyanidin B1
(***) quantified as mg/kg equivalents of Quercetin-3-O-glucoside

marblue/media solution (10/90 v/v) was added in each well.

Antioxidant activity

Cell oxidative stress was induced by tert-butyl hydroperoxide (TBH) on Caco-2 cells grown and differentiated in 24 well plates. Different concentrations of grape extracts (25-100 μg/ml) in MeOH solutions, or an equivalent volume of MeOH for the controls, were added to cells in PBS. After 30 min of incubation, TBH (2.5 mM) in aqueous solution was added and incubation was continued for another 2 h. The extent of oxidation was evaluated as malondialdehyde (MDA) formation, measured with the TBARS method (19).

Statistical analysis

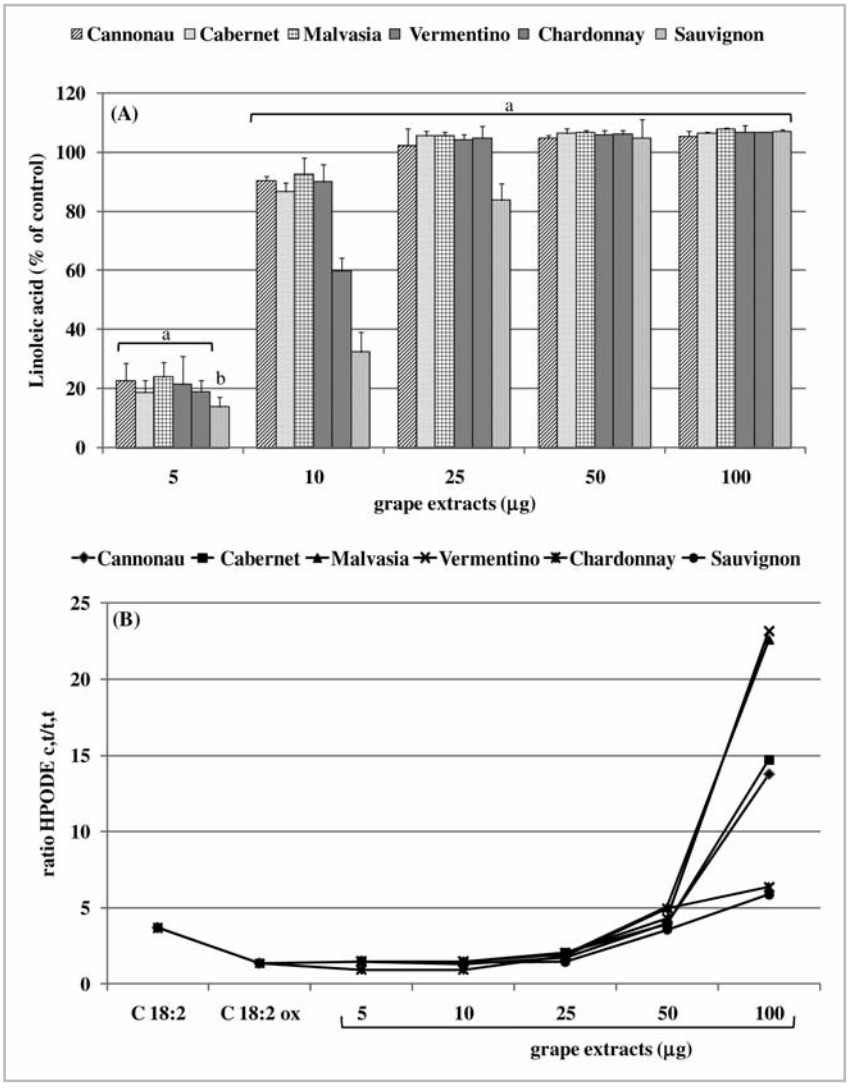
INSTAT software (GraphPad software, San Diego, CA) was

used to calculate the means and standard deviations of three independent experiments (n=6 or 9 for each sample/condition). One-way ANOVA was used to test whether the group means differed significantly.

Results and discussion

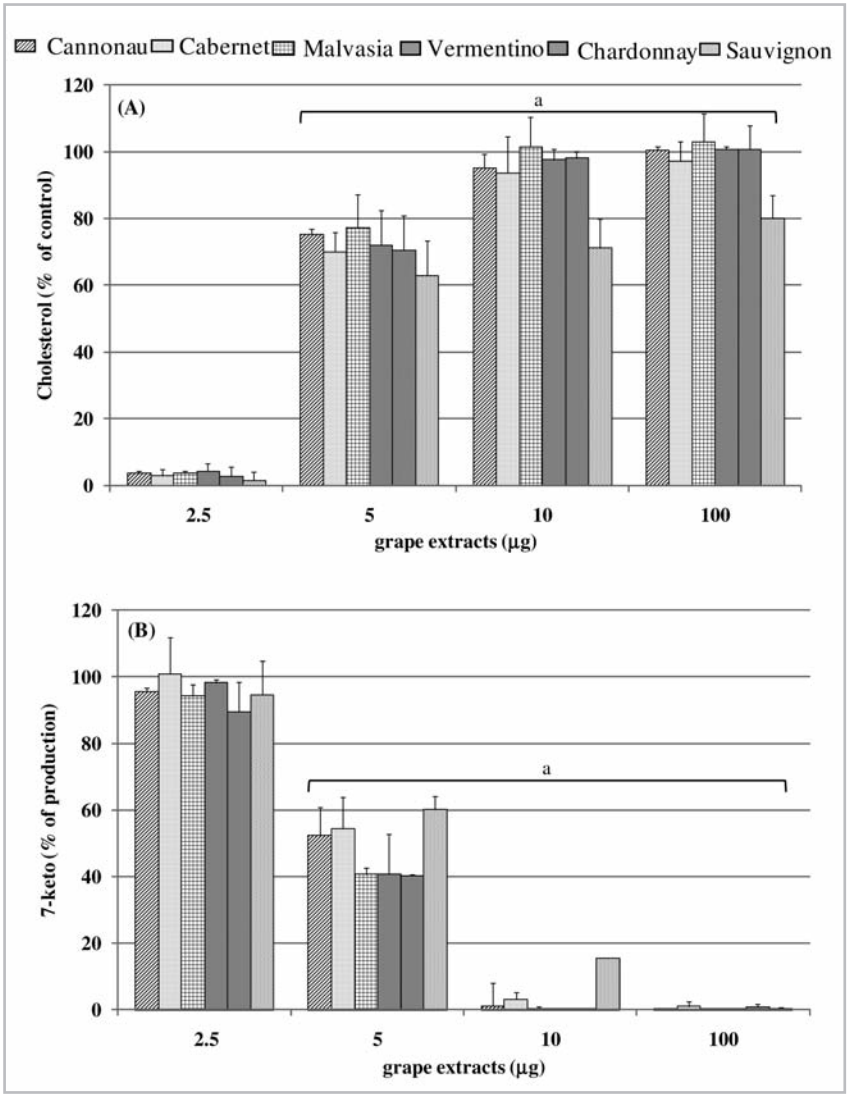
The present work was carried out on some grape extracts to test their potencies as antioxidants in simple in vitro lipid systems and in Caco-2 cells. The extracts were assayed for antioxidant activity in in vitro systems, during autoxidation of linoleic acid. During autoxidation, the oxidation pattern was followed by monitoring the consumption of linoleic acid and the formation of its major oxidation products, the HPODE isomers. Fig. 1A shows the results obtained during autoxidation of linoleic acid in the presence of different amounts of the extracts (5-100 μg). During autoxidation all the grape extracts showed a significant inhibition of the oxidative process from a concentration of 5 μg. The Sauvignon extract was active at higher concentrations, showing complete inhibition from a concentration of 50 μg. The Chardonnay extract gave 60% protection at 10 μg and total inhibition from 25 μg. The extracts of native cultivars, Cannonau, Malvasia and Vermentino, were the most effective, together with the Cabernet extract, showing 90% protection from a concentration of 10 μg and complete inhibition from 25 μg. The values of the HPODE isomers, c,t-9-HPODE, t,t-9-HPODE, c,t-13-HPODE and t,t-13-

Figura1 - Linoleic acid values, expressed as % of control (A), and ratio of the conjugated diene linoleic acid hydroperoxydes (c,t- and t,t-HPODE) (B) measured during the autoxidation of linoleic acid at 37°C for 32 h in the presence of different amounts (5-100 μg) of grape extracts. a=p<0.001, b=p<0.01 vs. controls



HPODE, were measured in all experimental systems. The two c,t isomers were added up, as well as the t,t isomers, and the c,t/t,t ratio was calculated for all tested compounds (Fig. 1B). The grape varieties native to Sardinia and the Cabernet grape, gave a clear shift versus the c,t isomers, showing increasing c,t/t,t ratios with increasing extract concentrations; the Chardonnay and Sauvignon extracts gave a slight shift of the c,t/t,t ratio from 50 μg. Our data showed that c,t/t,t ratio was shifted versus the c,t isomers, suggesting a mechanism of action involving hydrogen atom donation (20); the formation of HPODE isomers during the autoxidation process shows a paraboloid pattern (20), with an initial shift versus the c,t isomers that disappears with the fatty acid degradation, but is enhanced in the presence of a strong hydrogen atom donator. The extracts were assayed for antioxidant activity also during cholesterol autoxidation. The consumption of cholesterol and the formation of its major oxidation product, 7-keto, were measured as markers of the oxidative process. Fig. 2A shows data obtained during cholesterol oxidation in the presence of different amounts (2.5-100 μg) of the extracts. A significant inhibition of the oxidative process was observed for all extracts from a concentration of 5 μg. The Sauvignon extract showed an antioxidant activity lower than the other extracts, preventing the formation of the 7-keto (Fig. 2B) at the highest concentration tested; all the extracts prevented the total formation of 7-keto from a concentration of 10 μg (Fig. 2B).

Figura 2 - Cholesterol values, expressed as % of controls (A), and 7-keto formed, expressed as % of production (B), measured during the autoxidation of cholesterol at 140°C for 90 min in the presence of different amounts (5-100 μg) of grape extracts. a=p<0.001 vs. controls



Grape extracts were then tested in cell cultures to evaluate their activity. A preliminary set of experiments was performed to determine their non-toxic concentrations. Fig. 3 shows the percentage of Caco-2 cells viability after 24 h of incubation in the presence of the grape extracts (25-100 μg/ml) compared to the control: cell viability remained unchanged in the presence of extracts at all concentrations tested, demonstrating that the extracts by themselves do not induce significant toxicity. The activity of grape extracts was evaluated with regard to the oxidative damage induced in Caco-2 cells by TBH, and the extent of oxidative damage was measured as MDA production. In order to induce oxidative stress, differentiated Caco-2 cells were treated with TBH (2.5 mM for 2 h), the highest concentration able to induce a significant oxidative damage but not cell death (data not shown). A significant increase of MDA level, compared with controls, was observed in the culture medium of TBH treated cells (Fig. 4); pretreating the cells with the grape extracts of native cultivars and with the Cabernet extract a significant reduction of the MDA levels was observed starting with 25 μg/ml. Again the Chardonnay and Sauvignon extracts were active against oxidative damage from a higher concentration (50 μg/ml).

In conclusion our data show the effectiveness of grape extracts, from the concentration of 5 μg, in counteracting the oxidative damage in vitro lipid systems and in Caco-2 cells. The grape varieties native to Sardinia, in spite to the lower phenolic concentration, showed an antioxidant activity similar to that of the Cabernet extract,

Figura 3 - Percentage of cell viability, measured with the alamarblue assay, after 24 h exposure to different amounts (25-100 μg) of grape extracts

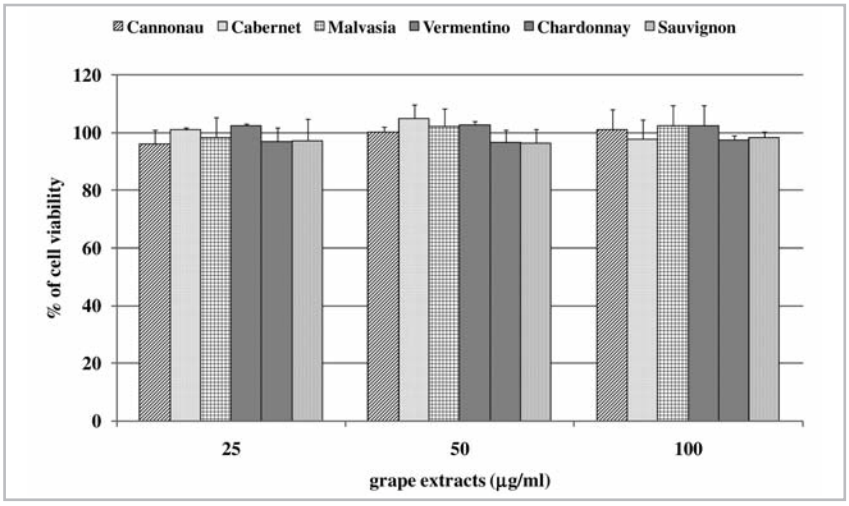
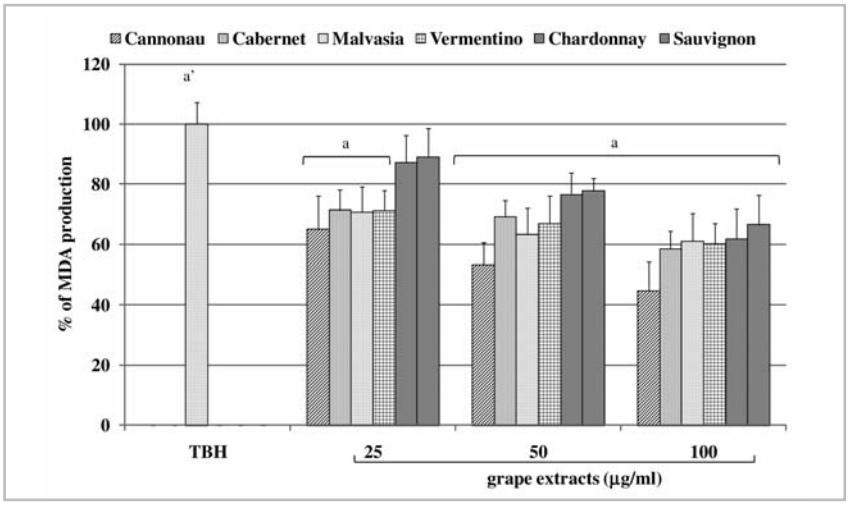


Figura 4 - Percentage of MDA production measured in the cells supernatants, pretreated with the different amounts (25-100 μg) of grape extracts and treated with TBH 2.5 mM. a'=p<0.001 vs. controls, a=p<0.001 vs. TBH treated



the richest in phenolic compounds, mainly anthocyanins, supporting the hypothesis that antioxidant activity of a phenolic extract is related not only to the total amount of phenols, but also to the relative proportions among the different classes of compounds, being each characterized by a peculiar activity that may be modulated depending on the reaction conditions. Data obtained in this work may contribute to the promotion of native varieties and are the basis for future research; native grapes extracts will be tested under different experimental conditions that will help to clarify the mechanism of action, and their activity will be evaluated in further investigations in vivo.

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