Food and Chemical Toxicology 127 (2019) 127–134

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Comparative analysis of chemical composition, antioxidant and anti-proliferative activities of Italian *Vitis vinifera* by-products for a sustainable agro-industry

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ARTICLE INFO

Keywords:
Vitis vinifera leaves
Waste
HPLC
Anti-proliferative
Antioxidant

ABSTRACT

Vitis vinifera leaves are wine industry wastes. In this study, the chemical composition, antioxidant and anti-proliferative activity of six Italian grapevine leaves extracts (Arvino, Gaglioppo, Greco Nero, Magliocco Canino, Magliocco Dolce, and Nocera) were evaluated. HPLC analyses revealed quercetin as dominant constituent (127.52–187.33 mg/kg) followed by rutin (55.99–143.67 mg/kg). The antioxidant activity was determined using DPPH, ABTS, FRAP and β-carotene bleaching tests. Gaglioppo showed the highest radical scavenging ability with IC_{50} of 7.2 and 19.1 μg/mL, for DPPH and ABTS, respectively. Magliocco Dolce showed a 1.6-times higher FRAP activity than that of the positive control BHT. The anti-proliferative activity was determined by SRB assay against MCF-7, MDA-MB-231, A549 and COR-L23 human tumor cells. Greco Nero showed the highest anti-proliferative activity against MDA-MB-231 with IC_{50} of 28.4 μg/mL. Based on the obtained results grape leaves should be considered an interesting ingredient for the development of functional food products.

1. Introduction

In Europe, agricultural waste is estimated in the order of 250 million tons *per* year. At global level, the amount of waste produced by the agro-food industries is around 800,000 tons *per* year, which represents a significant potential for the development of the bioenergy industry (Ayala-Zavala et al., 2010). Nowadays, there is a growing interest in finding new sources of functional ingredients starting from by-products of traditionally underestimated vegetable foods. Peels, seeds, shanks, leaves, wastewater, and unusable pulp represent more than 40% of total plant food (Goñi and Hervert-Hernández, 2011). These by-products are very rich in nutrients such as sugar, minerals, organic acids, dietary fibers and bioactive compounds, such as polyphenols and carotenoids, and could therefore be reused and have their own market (Sanchez-Zapata et al., 2009), assuming a relevant economic and scientific value in various industrial sectors, including the food, nutraceutical, pharmaceutical and cosmetic ones.

Vitis vinifera L. is a climbing shrub with large leaves belonging to the Vitaceae family, originally from Asia Minor and subsequently introduced to Europe and other continents. The inflorescence comes in the form of a bunch while the fruits are in the form of berries, whose color varies from green to purple-black. Grape leaves are used in the Mediterranean area and in particular in Greece.

It can be stuffed with meats, rice, vegetables, cheeses, nuts, dried fruits and spices. Fresh grape leaves must be blanched in hot water or a brine solution of salt and water to create an edible and flexible product (Katalinić et al., 2013; Alexiadou, 2017).

Several *in vivo* and *in vitro* studies have been carried out on *V. vinifera* and its by-products. *V. vinifera* leaves showed to exert multiple biological activities including antioxidant, antimicrobial (Abed et al., 2015), antidiabetic (Akabery and Hosseinzadeh, 2016), anti-hypercholesterolemic (Devi and Singh, 2017), anti-inflammatory and anti-tumor (Nassiri-Asl and Hosseinzadeh, 2016). These effects are due to the action of bioactive compounds, such as tannins, flavonoids, anthocyanins as well as organic acids and vitamins detected in the leaves of this plant (Hmamouchi et al., 1997).

Antioxidant activity is one of the most important properties of natural compounds with particular references to phenols. Oxidative

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https://doi.org/10.1016/j.fct.2019.03.007
Received 16 January 2019; Received in revised form 8 March 2019; Accepted 10 March 2019
Available online 13 March 2019

stress causes the alteration of biological macromolecules, such as lipids, proteins and nucleic acids and is considered as the factor responsible for the onset of numerous diseases including cancer (Carocho and Ferreira, 2013). In fact, in many types of cancer, high levels of reactive oxygen species (ROS) have been detected which, through different mechanisms of action, promote the development and progression of the disease (Liou and Storz, 2010). Endogenous defense mechanisms, represented by antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, work by neutralizing the action of ROS. The excessive production of ROS, compared to the antioxidant capacity of endogenous systems, determines the progression of oxidative stress leading to the onset of serious diseases, including cancer (Ozden et al., 2009).

Grape phenolic compounds, as natural antioxidants, act as scavengers of free radicals and ROS quenchers are able to interfere with the systems involved in the production of ROS, thereby blocking the progress of oxidative stress (Xia et al., 2010).

Over the years, several studies have shown the importance of natural bioactive compounds as anticancer agents, preventive of various chronic diseases (Mondal et al., 2012). Therefore, there is a growing interest in finding new sources of natural antioxidants that could be used as a preventive for many diseases. In this context, we have screened the chemical profile, antioxidant and anti-proliferative activity of six native Calabrian varieties of *V. vinifera* leaves in order to highlight their potential in the development of new functional food and nutraceutical products to identify new opportunities for the use of waste from the wine industry so far to be poorly considered.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Co. Ltd (Milan, Italy) and VWR International (Milan, Italy) and, unless specified otherwise, were analytical grade or higher. Cell culture and cell culture materials were obtained from Sigma-Aldrich Chemical Co. Ltd (Milan, Italy).

2.2. Plant materials and extraction procedure

V. vinifera leaves of six varieties of native Calabrian vines have been analyzed. The grapevine varieties were Arvino, Gaglioppo, Greco Nero, Magliocco Canino, Magliocco Dolce and Nocera. Leaves were collected in September 2017 from a local producer (Azienda Agricola Donna Fidelia, Belvedere Marittimo, Cosenza, Southern Italy, (Latitude 39° 38′ 11 N; Longitude 15° 50′ 40 E). Plant materials were subjected to ultrasound assisted extraction procedure using an ultrasonic water-bath (Branson model 3800-CPXH, Milan, Italy). Briefly, 250 mL of a hydroalcoholic solution (EtOH/H_2O 50:50 v/v) were used for the extraction of fresh leaves (50 g). For each sample, three extraction cycles with an ultrasonic frequency of 40 kHz for 30 min were carried out. Then, the mixture was filtered under vacuum through Whatman filter, and the solvent was removed with a rotary vacuum evaporator at 30 °C. Samples were stored at −20 °C until analysis.

2.3. Total phenols content

The total phenols content (TPC) was determined using Folin-Ciocalteu method (Gao et al., 2000). Folin-Ciocalteu reagent is a mixture in aqueous solution of phosphomolybdate and phosphotungstate. Firstly a sample stock is prepared, adding 5 mL of methanol to 7,5 mg of extract. Briefly, 100 μL of stock was mixed with 0.5 mL Folin-Ciocalteu reagent, 1 mL of distilled water and 1.5 mL of 20% Na_2CO_3. It was done in triplicate. After 2 h incubation at 25 °C the absorbance was measured at 765 nm using a Perkin Elmer 40 UV-VIS spectrophotometer. The total content of phenols was expressed in mg equivalent of chlorogenic acid *per* g fresh weight (FW).

2.4. Total flavonoids content

V. vinifera leaves total flavonoid content (TFC) was determined using a method that uses $AlCl_3$ (Loizzo et al., 2012). The same stock of polyphenols was used, done in triplicate. One mL of extract solution was added to 1 mL of 2% aluminum chloride solution. It was allowed to incubate at room temperature for 15 min and read at 510 nm with a Perkin Elmer 40 UV-VIS spectrophotometer. Quercetin was chosen as the standard and the total flavonoid content was expressed in *per* g fresh weight (FW).

2.5. Total anthocyanins content

The total anthocyanins content (TA) was determined using the differential pH method (Wrolstada et al., 2005). Anthocyanins undergo a reversible modification of the structure with a change in the pH that occurs with a variation in the absorbance spectrum. Seven and half mg of extract were added to 5 mL of distilled water. For each sample two dilutions were prepared, one with a 0.025 M hydrochloric acid buffer solution at pH 1, and the other with a 0.4 M sodium acetate buffer solution at pH 4.5, corrected with hydrochloric acid. The solutions were left to equilibrate for 15 min. Spectrophotometric reading was performed at 510 nm and 700 nm. The results were expressed as equivalent mg of cyanidine-3-*O*-glucoside *per* 100 g fresh weight (FW).

2.6. High performance liquid chromatography/diode array detector (HPLC/DAD) analysis

High performance liquid chromatography coupled to a diode array detector (HPLC/DAD) was used to determine the phenolic profile of the extracts. The analysis was performed on a Knauer system (ASI - Advanced Scientific Instruments, Berlin, Germany) equipped with two Smartiline Pump 1000 pumps, a Rheodyne injection valve (20 μL) and a UV–VIS photodiode series detector equipped with a semi-microcell. The antioxidant compounds were separated on a TSK gel ODS-100 V column (TOSOH Bioscience, Germany) (250 × 3.0 mm; 3 μm). The temperature of the column was 30 °C with a flow rate of 0.5 mL/min. The mobile phase consisted of water/formic acid (99.9: 0.1, v/v solvent A) and acetonitrile/formic acid (99.9: 0.1, v/v; solvent B). The separation was carried out according to the following gradient: 0.01–20.00 min, 5% B isocratic; 20.01–50.00 min, 5–40% B; 50.01–55.00 min, 40–95% 120 B; 55.01–60.00 min, 95% B isocratic. The identification and quantification of the antioxidant compounds was performed by comparing the spectra and relative retention times of the sample peaks with those obtained by injecting pure standards, i.e. gallic acid, catechin, caffeic acid, syringic acid, rutin, *trans*-resveratrol, polydatin and quercetin that are chosen as markers. The survey was performed at the wavelengths of 280, 254, 330 and 305 nm. Data processing was performed using Clarity Software (Chromatography Station for windows). Extracts were dissolved in 10 mL of methanol and filtered through a 0.45 μm millipore filter (GMF Whatman) before the HPLC/UV–Vis determination. The results were expressed as mean ± SD of three determinations. With this method, the following compounds have been identified and quantified: gallic acid, (+) - catechin, caffeic acid, syringic acid, rutin, myricetin, *trans*-resveratrol, polydatin and quercetin.

2.7. Evaluation of antioxidant activity

Several methods have been developed to determine the antioxidant activity of samples; the most frequently used are *in vitro* methods based on capturing or scavenging free radicals generated in the reaction or in the reduction of metal ions. In this work three methods (DPPH, β-carotene bleaching and FRAP) that measure different types of antioxidant function were applied.

2.7.1. ABTS and DPPH radical scavenging assays

The radicals scavenging potential was investigated by using two different spectrophotometric methods, namely 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.

A DPPH solution in ethanol (0.25 mM) was mixed with *V. vinifera* leaves extracts in ethanol at different concentrations ranging from 31.5 to 1000 mg/mL. The bleaching of DPPH was determined spectrophotometrically at 517 nm. A solution of ABTS radical cation was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulphate and stored at room temperature for 12 h before use. Then, it was diluted with ethanol to an absorbance of 0.70 at 734 nm. After addition of extracts in ethanol at concentrations ranging from 5 to 80 mg/mL to 2 mL of diluted $ABTS^+$ solution, absorbance was measured at 734 nm. The radicals (ABTS or DPPH) scavenging ability was calculated as follows: scavenging activity = $[A_0 - A)/(A_0] \times 100$, where A_0 is the absorbance of the control reaction and A is the absorbance in the presence of the extract (Tundis et al., 2017). Ascorbic acid was used as positive control in both assays.

2.7.2. β-Carotene bleaching test

The protection of extract for lipid peroxidation was measured as previously described (Tundis et al., 2017). Briefly, β-carotene solution was added to linoleic acid and 100% Tween 20. The absorbance of the samples, standard and control was measured at 470 nm against a blank at t = 0 and successively after 30 and 60 min of incubation.

2.7.3. FRAP (Ferric Reducing Ability Power) assay

The FRAP assay was applied following the procedure previously described (Loizzo et al., 2016). The FRAP value represents the *ratio* between the slope of the linear plot for reducing Fe^{3+}-TPTZ reagent by different Colombian fruits extract compared to the slope of the plot for $FeSO_4$.

2.7.4. Relative Antioxidant Capacity Index (RACI) calculation

Relative Antioxidant Capacity Index (RACI) is a statistical tool that allows determining the antioxidant capacity of food matrices. It is the average value that is generated by integrating the data obtained from TA, TFC, TPC, ABTS, DPPH, FRAP and β-carotene bleaching tests of each sample (Sun and Tanumihardjo, 2007). Standard scores were derived from data from different chemical methods without unrestricted units and no variance between the methods. The standard score is calculated using the following equation:

$$RACI = (x - \mu)/\sigma$$

where x is the raw data, μ is the mean, and σ is the standard deviation.

2.7.5. Global Antioxidant Score (GAS)

Global Antioxidant Score (GAS) is a correlation index of the results obtained from the different *in vitro* assays that allows to evaluate the total antioxidant activity of the samples being analyzed. For each sample the average of five T-scores is taken into account for the GAS value between zero and three. T-score is calculated by the following equation: $T - score = (X - min)/(max - min)$, where min and max, respectively, represent the smallest and largest values of variable X among the investigated extract (Leeuw et al., 2014).

2.8. Anti-proliferative activity

In this study four cancer cell lines namely human Caucasian breast carcinoma (MCF-7, ECACC N°:86012803), human Caucasian breast adenocarcinoma (MDA-MB-231, ECACC N°:92020424), lung carcinoma A549 (ECACC N°:86012804) and human Caucasian lung large carcinoma COR-L23 cells (ECACC N°:92031919) were used. Prior to use, all media, buffers, trypsin and dyes were filter-sterilized and warmed to 37 °C. The COR-L23 cells were cultured in RPMI 1640 medium, while MCF-7, MDA-MB-231, and A549 cells were cultured in DMEM. Both media were supplemented with 10% foetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. The cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. Cells trypsinization was done using a 1:30 dilution of standard Trypsin-EDTA solution. Cells counting and viability were performed using a standard trypan blue cell counting technique.

The anti-proliferative activity of the extracts was determined through an *in vitro* assay that allows to evaluate the inhibition of cell growth, using a bright pink amino-xanthous dye, sulforodamine B (SRB) (Loizzo et al., 2009). It is therefore a colorimetric assay, through which the number of cells can be indirectly estimated. Cells were trypsinized, counted and placed in 96-well plates. Optimal plating density of each cell line was determined over a range $5–15 \times 10^4$ to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 490 nm and cell number where analyzed by the SRB assay, and incubated to allow for cell attachment. After 24 h the cells were treated with serial dilutions of the samples. Each sample was initially dissolved in DMSO and further diluted in medium to produce different concentrations. One hundred microliters/well of each dilution were added to the plates in six replicates to obtain the final concentrations ranging from 5 to 200 μg/mL for the sample. The final mixture used for treating the cells contained not more than 0.5% of the solvent (DMSO), the same as in the solvent-control wells. After 48 h of exposure 100 μL of ice-cold 40% trichloroacetic acid was added to each well, left for 1 h at 4 °C, and washed with distilled water. The trichloroacetic acid -fixed cells were stained for 30 min with 50 μL of 0.4% (w/v) SRB in 1% acetic acid. Plates were washed with 1% HOAc and air dried overnight. For plate reading, the bound dye was solubilised with 100 μL of 10 mM tris base (tris[hydroxymethyl]aminomethane). The absorbance of each well was read on a Molecular Devices SpectraMax Plus Plate Reader (Molecular Devices, Celbio, Milan, Italy) at 490 nm. Cell survival was measured as the percentage absorbance compared to the untreated control. Vinblastine sulfate salt and taxol were used as positive control. The anti-proliferative activity of *V. vinifera* leaves extracts was expressed in terms of IC_{50} values.

2.9. Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± S.D. Differences were evaluated by the one-way analysis of variance (ANOVA) test completed by a multicomparison Dunnett's test ($\alpha = 0.05$). The inhibitory concentration 50% (IC_{50}) was calculated by a nonlinear regression curve with the use of Prism Graphpad prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA (www.graphpad.com). The concentration-response curve was obtained by plotting the percentage of inhibition *versus* the concentrations. PCA was applied to examine the relationships between the chemical constituents of the leaves using the SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). The results are presented in terms of loading and score plots.

3. Results and discussion

3.1. Chemical composition of calabrian V. vinifera leaves extracts

It is well known that phenols and flavonoids are the important antioxidant substances contained in most natural plants. Genotypes, environmental factors and postharvest processing conditions influence the amount of bioactive compounds present in the grapevine. Herein, the TPC, TFC and TA content of *V. vinifera* leaves extracts were evaluated (Table 1). The TPC content ranged from 39.11 to 294.54 mg chlorogenic acid equivalents *per* g plant material, respectively for Magliocco Canino and Greco Nero. A TFC in the range 2.11–26.16 mg quercetin

Table 1
Extraction yield, total content of phenols, flavonoids and anthocyanins of different Calabrian *V. vinifera* leaves extracts.

Sample	Extraction Yield[a]	TPC[b]	TFC[c]	TAC[d]
G	4.4	111.7 ± 1.9	26.2 ± 1.2	0.1 ± 0.04
MD	4.2	87.6 ± 1.2	23.9 ± 1.0	0.9 ± 0.05
MC	4.1	39.1 ± 1.0	11.1 ± 0.9	0.4 ± 0.09
A	6.3	200.6 ± 2.2	2.6 ± 0.4	0.7 ± 0.07
GN	7.9	294.5 ± 2.5	2.1 ± 0.5	0.4 ± 0.04
N	6.2	201.6 ± 2.1	2.2 ± 0.4	0.9 ± 0.06
		**	**	**

G: Gaglioppo; MD: Magliocco Dolce; MC: Magliocco Canino; A: Arvino; GN: Greco Nero; N: Nocera. TPC: Total phenols content; TF: Total flavonoids content; TA: Total antocyanins content.
[a] : %.
[b] mg Chlorogenic acid equivalents *per* g FW.
[c] mg Quercetin equivalents *per* g FW.
[d] mg Cyanidin-3-*O*-glucoside equivalents *per* g FW.

equivalents *per* g plant material was found in Greco Nero and Gaglioppo, respectively. Both TPC and TFC content are in the same order of magnitude as confirmed by Essa et al. (2017). However, as reported by Güler et al. (2014) the TPC and TFC content significantly varied in the six investigated varieties.

Anthocyanins play a crucial role in the color of grapes and consequently in wine. Leaves TA content ranged from 1.10 to 0.95 mg cyaniding-3-*O*-glucoside equivalents *per* 100 g FW for Gaglioppo and Nocera varieties, respectively. High levels of phenolic compounds in leaves extracts have been found in several studies (Katalinić et al., 2013), however it is difficult to compare our data with those reported in literature, since different units were used to express the obtained results.

The phenolic profile by HPLC lead to the identification of selected markers gallic acid, (+)-catechin, caffeic acid, syringic acid, rutin, *trans*-resveratrol, polydatin, myricetin and quercetin (Table 2, Supplementary material). Quercetin represent the most abundant phenolic compound with concentration in the range 127.52–187.33 mg/kg for Nocera and Greco Nero varieties, respectively followed by rutin, with concentration ranging from 55.99 to 143.67 mg/kg for Arvino and Magliocco Dolce leaves extracts, respectively. Myricetin was also detected with concentration ranging from 3.8 to 6.7 mg/kg for Arvino and Magliocco Dolce, respectively. Arvino leaves extract showed a higher *trans*-resveratrol content (26.63 mg/kg) in comparison to other investigated samples. Among hydroxybenzoic acids, syringic acid was the most representative with concentration in the range from 108.37 to 186.86 mg/kg, for Gaglioppo and Arvino variety, respectively. Greco Nero variety showed the highest content of (+)-catechin (68.4 mg/kg) followed by Arvino grapes (56.9 mg/kg).

The phenolic content of different varieties of *V. vinifera* leaves is dependent and strongly influenced by the sample collection period. Katalinić et al. (2013), showed a significant increase in flavonols content, particularly for myricetin and quercetin, in September leaves compared to May leaves with concentration ranging from 10 to 35 mg/kg of dry leaves, depending on the variety and the time of sampling. Myricetin and quercetin are two of the main representative compounds in leaves from Narince, Saruhanbey, Sultani Çekirdeksiz, Sultan1, and Sultan7 Turkish grape cultivars (Güler and Candemir, 2014). Calabrian grapes cultivars are characterized by a higher *trans*-resveratrol content in comparison to other cultivars. Balík et al. (2008) reported a *trans*-resveratrol content ranging from 2.5 to 8.5 mg/kg for André and Saint Laurent and Blauer Portugieser grapes, respectively. It is interesting to note that these cultivars did not contain (+)-catechin.

3.2. Antioxidant capacity of the grapevine leaves extracts

Herein, we reported the antioxidant potential of leaves extract from six grapevines from Calabria region. To evaluate the antioxidant activity of the samples, four *in vitro* assays (ABTS, DPPH, β-carotene bleaching, and FRAP), normally used to determine the antioxidant potential of plant extract and food matrix, were applied. These assays are based on an electronic transfer reaction, such as FRAP, DPPH and ABTS test or on a transfer reaction of a hydrogen atom, such as β-carotene bleaching inhibition assay (Huang et al., 2005). Data are reported in Table 3. Gaglioppo leaves extract showed the highest radical scavenging activity with IC_{50} values of 7.19 and 19.12 μg/mL for DPPH and ABTS, respectively, whereas Magliocco Dolce resulted to be more active against DPPH· radical with IC_{50} value of 12.47 μg/mL. The bleaching of β-carotene is the consequence of hydro-peroxides formation from linoleic acid. The assay is based on the ability of the phytochemicals with antioxidant activity to reduce the oxidation of linoleic acid and to inhibit the free radicals generated by the emulsion system (Koleva et al., 2002). The best protection of lipid peroxidation was observed with Arvino and Nocera leaves extracts, with IC_{50} values of 41.80 and 43.34 μg/mL, respectively. The antioxidant capacity of the various samples was also evaluated using the FRAP method. In this case the evaluation of the reducing power is related to the ability of the sample to reduce the ferric iron to ferrous (from Fe^{3+} to Fe^{2+}). Extracts were tested at concentration of 2.5 mg/mL and butylhydroxytoluene (BHT) was used as a positive control. A ferric reducing power 1.5-times higher than that of BHT was found with Magliocco Dolce leaves extract that showed a FRAP value of 100.41 μM Fe (II)/g. A significant result was also obtained with Greco Nero (93.35 μM Fe (II)/g). The observed reducing activity was noteworthy since in cells, the presence of Fe^{+2} is toxic since this ion could react in the Fenton reaction with H_2O_2 to generate OH· that will initiate the oxidation (Halliwell, 2008).

Our data are in agreement with those reported by Katalinić et al. (2009) who found a DPPH radical scavenging ability of Croatian *V. vinifera* leaves extracts with IC_{50} value of 61.69 and 70.32 μg/mL in the May and September leaves, respectively. Radical scavenging potential was observed with the ABTS test with percentage of inhibition of 59.36 and 71.38 μg/mL in the May and September leaves, respectively. Orhan et al. (2007) evaluated the antioxidant activities of four fractions of *V. vinifera* leaves using the DPPH assay, demonstrating an effective DPPH radical scavenger activity. The fraction in EtOAc showed the most

Table 2
Determination of total phenols, flavonoids and anthocyanins content and relative determination by HPLC-DAD of Gaglioppo, Magliocco Dolce and Canino, Arvino, Greco Nero and Nocera leaves extracts (mg/Kg).

Sample	Gallic acid	(+)-Catechin	Caffeic acid	Syringic acid	Rutin	Quercetin	Mirycetin	trans-Resveratrol	Polydatin
G	3.5 ± 0.07	55.2 ± 0.17	3.6 ± 0.07	108.37 ± 0.22	115.3 ± 0.22	128.7 ± 0.24	5.2 ± 0.06	16.6 ± 0.10	77.4 ± 0.20
MD	1.5 ± 0.08	27.0 ± 0.18	3.4 ± 0.05	112.36 ± 0.17	143.7 ± 0.37	136.8 ± 0.25	6.7 ± 0.09	12.2 ± 0.05	50.1 ± 0.17
MC	1.2 ± 0.04	34.4 ± 0.23	5.5 ± 0.05	177.75 ± 0.33	119.0 ± 0.40	142.6 ± 0.31	5.8 ± 0.08	22.0 ± 0.11	42.4 ± 0.30
A	2.9 ± 0.11	56.9 ± 0.28	4.1 ± 0.07	186.86 ± 0.37	55.9 ± 0.33	153.4 ± 0.27	3.8 ± 0.06	26.6 ± 0.08	75.9 ± 0.18
GN	2.1 ± 0.07	68.4 ± 0.31	3.7 ± 0.04	139.74 ± 0.17	73.8 ± 0.28	187.3 ± 0.30	4.0 ± 0.02	17.9 ± 0.09	56.3 ± 0.22
N	2.0 ± 0.04	33.9 ± 0.22	4.6 ± 0.08	157.11 ± 0.20	87.5 ± 0.18	127.5 ± 0.20	5.1 ± 0.06	21.9 ± 0.07	52.9 ± 0.27
	**	**	**	**	**	**	**	**	**

G: Gaglioppo; MD: Magliocco Dolce; MC: Magliocco Canino; A: Arvino; GN: Greco Nero; N: Nocera.

Table 3
Antioxidant activity of Gaglioppo, Magliocco Dolce and Canino, Arvino, Greco Nero and Nocera leaves extracts and related RACI and GAS.

Sample	DPPH test	ABTS	FRAP test	β-Carotene bleaching test		RACI	GAS
	IC_{50} (μg/mL)	IC_{50} (μg/mL)	μM Fe(II)/g[a]	IC_{50} (μg/mL)			
				30 min	60 min		
G	7.19 ± 0.8	19.12 ± 1.6****	81.36 ± 2.8***	28.15%[b]	23.72%[b]	−0.33	0.43
MD	12.47 ± 0.9**	23.80 ± 1.9****	100.41 ± 4.6****	43.36%[b]	25.29%[b]	−1.64	1.14
MC	35.30 ± 2.4****	31.02 ± 2.5****	67.06 ± 3.5	34.85%[b]	29.30%[b]	−0.11	0.57
A	32.99 ± 1.8****	86.33 ± 4.7****	86.56 ± 2.7****	41.80 ± 1.7****	45.70 ± 1.9****	0.29	3.39
GN	77.88 ± 3.4****	78.85 ± 3.5****	93.35 ± 3.9****	50.76%[b]	41.02%[b]	0.23	2.68
N	30.28 ± 1.9****	69.47 ± 2.9****	80.64 ± 3.9***	43.34 ± 1.8****	95.22 ± 3.6****	0.72	3.48
Positive control							
Ascorbic acid	5.0 ± 0.8	1.7 ± 0.4					
BHT			63.2 ± 4.3				
Propyl gallate				1.0 ± 0.04	1.0 ± 0.06		

G: Gaglioppo; MD: Magliocco Dolce; MC: Magliocco Canino; A: Arvino; GN: Greco Nero; N: Nocera. [a]: at the concentration of 2.5 mg/mL [b]: sample tested at 100 μg/mL; DPPH test: One-way ANOVA followed by a multicomparison Dunnett's test ($\alpha = 0.05$): ****$p < 0.0001$, **$p < 0.05$ compared with ascorbic acid. Antioxidant Capacity Determined by Radical Cation (ABTS+): One-way ANOVA followed by a multicomparison Dunnett's test ($\alpha = 0.05$):****$p < 0.0001$ compared with ascorbic acid. Ferric Reducing Ability Power (FRAP): One-way ANOVA followed by a multicomparison Dunnett's test ($\alpha = 0.05$): ****$p < 0.0001$, ***$p < 0.001$ compared with BHT. β-Carotene bleaching test 30 and 60 min incubation: One-way ANOVA followed by a multicomparison Dunnett's test ($\alpha = 0.05$): ****$p < 0.0001$ compared with propyl gallate.

activity, with an inhibition of 92.8%, followed by the fraction in $CHCl_3$, with a percentage of 41.4%. Recently, Katalinić et al. (2013) reported the antioxidant potential of extracts from six *V. vinifera* varieties. Leaves collected in August showed an average FRAP value similar to those obtained in our study, in a range of 79.7–118.4 mM Trolox equivalent for Marastina and Vranac varieties, respectively. All investigated samples showed EC_{50} values higher than those found for Calabrian leaves extract. This observation confirmed that grape leaves antioxidant activities are affected by several factors including variety, country of cultivation and the climatic conditions.

In determining the antioxidant properties of the food matrix, the combined effects of the bioactive components should be considered. The RACI value was calculated for all the samples under study as the average of the standard scores transformed from the raw data generated with different antioxidant tests without differences in units and variances. Each test contributed the same weight in building RACI. Reported positive values of RACI equal to 0.72, 0.29 and 0.23, respectively in the extracts of Nocera, Arvino and Greco Nero, confirmed the previous values obtained from antioxidant tests. Data obtained from the DPPH, ABTS, FRAP and β-carotene bleaching tests were used to calculate, for each sample, the value of GAS that is used to compare the antioxidant power of the extracts. It was observed that the extracts of Gaglioppo and Magliocco Canino have the lowest GAS value, equal to 0.43 and 0.57, showing the highest antioxidant power. Therefore, all grape leaves exhibited high levels of natural antioxidants. The antioxidant activity of *V. vinifera* leaves was confirmed *in vivo*. Devi and Singh (2017) demonstrated that the methanol and aqueous extract of *V. vinifera* increase serum reduced glutathione (GSH) level and serum catalase level. The significant increase in serum GSH suggested that *V. vinifera* leaves extract acts by an indirect pathway that one or more phytochemicals are able to influence GSH production and/or reduction process of GSSG to GSH. The high level of GSH after *V. vinifera* leaves administration is important also because it contributes to the chemoprevention.

Phenolic compounds exert antioxidant activity through different mechanisms of action, including the direct extinction of ROS, by the inhibition of enzymes and the chelation of metal ions like Fe^{3+} and Cu^{+} and by inhibition of oxidative chain reactions.

According to Katalinić et al. (2013), the radical scavenging activity evaluated by the DPPH and ABTS tests revealed a positive *Pearson's* correlation coefficient with total phenol content with r values of 0.72 and 0.85, respectively. Correlation analysis revealed, also, that the total carotenoid content positively correlated with β-carotene bleaching test (r values of 0.59 and 0.65 at 30 and 60 min incubation, respectively). Among phytochemicals identified in our samples, a positive correlation was observed for quercetin and *trans*-resveratrol with r values of 0.91 and 0.56, and 0.66, and 0.72 for DPPH and ABTS, respectively. The stilbene compound also positively correlated with β-carotene bleaching test evaluated after 30 min incubation ($r = 0.65$).

3.3. Anti-proliferative activity

The anti-proliferative activity of *V. vinifera* leaves extracts on four tumor cell lines (A549, COR-L23, MDA, MCF-7) was evaluated. Data are reported in Table 4. Analysis of data evidenced that Greco Nero leaves extract showed a promising anti-proliferative activity against MDA/ADR cell line with IC_{50} value of 28.38 μg/mL followed by Gaglioppo leaves extract (IC_{50} value of 68.2 μg/mL). A lower activity was observed in MCF-7 cells where Magliocco Dolce showed the higher anti-proliferative activity with IC_{50} value of 148.2 μg/mL followed by Magliocco Canino (IC_{50} value of 156.6 μg/mL). Except Nocera sample, all investigated extracts inhibited lung carcinoma A549 cells in a concentration-dependent manner. In particular, Gaglioppo and Greco Nero samples exhibited IC_{50} values of 96.4 and 102.7 μg/mL. These values are 0.7-times higher than that reported for the vinblastine used as positive control.

From the analysis of the results it is possible to highlight that all investigated samples at maximum concentration tested were unable to have an effect on 3T3L1 cells used as control cells. This inactivity is probably due to selective action of *V. vinifera* phytochemicals in mechanisms that regulate cell proliferation. The anti-proliferative activity of different varieties of *V. vinifera* leaves extracts in different cancer cells was previously investigated. Chakraborty et al. (2016) reported the moderate anticancer activity against osteocarcoma cells MG63 of aqueous and methanol grape leaves extracts.

Abed et al. (2015) evaluated the effects of grape leaves extracts collected from two locations in Palestina against lung cell carcinoma A549. The better IC_{50} values of 90 and 85 μg/mL were recorded for Baituni variety collected in Beit Omar and Dahria, respectively, in comparison to Shami variety extract collected in the same place (IC_{50} values of 140 and 165 μg/mL, respectively).

The efficacy of different grape derived products including stem,

Table 4
Anti-proliferative activity (IC_{50} $\mu g/mL$) of Gaglioppo, Magliocco Dolce e Canino, Arvino, Greco Nero and Nocera leaves extracts.

Sample	MCF-7	A549	MDA-MB-231	COR-L23	3T3L1
G	170.5 ± 2.1****	102.7 ± 1.9****	68.2 ± 1.5****	12.9%[a]	> 200
MD	148.2 ± 2.0****	145.9 ± 2.3****	92.6 ± 2.4****	5.8%[a]	> 200
MC	156.6 ± 2.5****	131.6 ± 2.0****	95.8 ± 2.6****	5.9%[a]	> 200
A	> 200	> 200	38.1%[a]	16.6%[a]	> 200
GN	13.6%[a]	96.4 ± 1.7****	28.4 ± 1.2****	> 200	> 200
N	20.5%[a]	> 200	41.8%	16.9%[a]	> 200
Positive control					
Vinblastine		67.3 ± 2.0		45.5 ± 1.9	37.6 ± 1.7
Taxol	0.1 ± 0.006		2.0 ± 0.5		

G: Gaglioppo; MD: Magliocco Dolce; MC: Magliocco Canino; A: Arvino; GN: Greco Nero; N: Nocera. MCF-7, human breast cancer cells; MDA-MB-231breast adenocarcinoma cells; A549, human lung carcinoma; COR-L23 lung large carcinoma. Data are obtained by nonlinear regression analysis of three independent experiments, with triplicate samples and are expressed as the mean ± SD ($n = 3$).
****$p < 0.0001$ compared with positive controls (Vinblastine and Taxol).
[a] Sample tested at 200 $\mu g/mL$. One-way ANOVA followed by a multicomparison Dunnett's test ($\alpha = 0.05$).

skins, seeds, grape pomace and lees against different cancer cell lines was largely investigated. Skins, seeds, grape pomace and lees alcoholic extracts from the Arcaş grape variety influenced the proliferation of cervical cancer cells in a concentration and time-dependent manner with the following trend: seed > grape pomace > lees. In particular, seed extract inhibited the development of HeLa cells with 40.89% after a treatment of 24 h, and 71.69% after a treatment of 48 h (Nechita et al., 2012).

Sahpazidou et al. (2014) used the SRB assay to investigate the anti-proliferative activity of several grapes stem extracts against colon cells (HT29), breast (MCF-7 and MDA-MB-231), renal cells (786–0 and Caki-1) and thyroid (K1) cancer cells. Generally, Voidomato grape variety exerted the highest anti-proliferative activity with IC_{50} values of 120.5 and 121 $\mu g/mL$ for MDA-MB-231 and MCF-7, respectively. A similar effect was also observed with Mavrotragano against hormone independent, ER negative breast carcinoma cells. A promising activity was also observed when kidney tumor cells are treated with Voidomato grape variety extract with IC_{50} value of 134 $\mu g/mL$.

The anticancer activity of grape products extracts should be attributed to the presence of high concentrations of bioactive compounds with particular reference to polyphenols as predominate phytochemicals, among them, rutin, quercetin, *trans*-resveratrol and myricetin. A positive Pearson's correlation coefficient was found for rutin and A549, MCF-7, MDA-MB-231 with r values of 0.79, 0.88, and 0.91, respectively. With regard to our tested cells, a perusal analysis of the literature revealed that rutin promotes the TNF-α-induced apoptosis in human lung carcinoma cells and it should be able to regulate the expression of GSK-3β protein in A549 cells (Wu et al., 2017). Differently, quercetin exerted its anticancer effect by the disassembling effect on mitotic apparatus with particular reference to actin depletion (Klimaszewska-Wiśniewska et al., 2017). Moreover, this dietary flavonoid induces apoptosis and cell cycle arrest *via* modification of Foxo3a signalling in triple-negative breast cancer cells (Nguyen et al., 2017). Among the predominant compounds of Calabrian grapes leaves extracts *trans*-resveratrol was also identified. This stilbene induced cell cycle arrest in S-phase and induction of γ-H2AX, which is a hallmark of DNA damage after UV irradiation in MDA-MB-231. Previously, Pozo-Guisado et al. (2002) showed that *trans*-resveratrol was able to induce apoptosis in MCF-7 cells. Resveratrol exert its anti-proliferative effect against A549 cells by a direct decreasing of rate proliferation and inducing cell cycle arrest and cell apoptosis as a consequence of enhancement of ROS production in cancer cells. Moreover, this stilbene compound inhibited lung cancer cells metastatic process (Yousef et al., 2017). A blockage of the lung cancer cell metastatic process as consequence of interference on ERK signalling pathway was also reported for myricetin (Shih et al., 2009). Moreover, Ci et al. (2018) demonstrated that this flavonoid decreased the activities of MMP-2/9 and mRNA levels of ST6GALNAC5 expression in breast cancer models. Analysis of data evidenced that although the myricetin concentration in Calabrian grape leaves extracts was moderate, positive r values of 0.66, 0.77, 0.80 for A549, MCF-7 and MDA-MB-231 could be calculated. Since the cytotoxic effect cannot be attributed to a single compound, a synergism between the different bioactive secondary metabolites should be considered (Lazzè et al., 2009). For the above-mentioned reason all the bioactive compounds found in high concentration in grape leave extracts are potentially useful candidates for combination therapy with conventional drugs acting as nucleic acid-directed agents or novel cytoskeletal-directed agents.

3.4. Principal Component Analysis

Results were analyzed by a multivariate PCA method in order to reduce the number of artificial variables (D'Agostino et al., 2014). According to the PCA results, four dimensions were necessary for complete explanation of the data variability. As can be seen, most of the variance in leaves are explained by PC1, PC2 and PC3 (Fig. 1). The first three components of the PCA showed 85% of the total variance (48.55% for component 1, 21.15% for component 2 and 15.29% for component 3). The fourth component (PC4) explained a small percentage, while, the successive PCs could be considered as not statistically significant.

The first component (PC1) has highly positively correlated with TCA, ABTS test, β-carotene bleaching test at 30 and 60 min of incubation, and negatively correlated with MCF7, A549, MDA-MB-231, TFC, rutin and myricetin. The second principal component (PC2) was found to be positively correlated with TPC, catechin, quercetin, DPPH and ABTS tests and negatively correlated with TFC, rutin, myricetin, MCF7 and MDA-MB-231. Finally, PC3 was found to be positively correlated with gallic acid, catechin, and polydatin and negatively correlated with TCA. The fourth principal component (PC4), showed a high positive correlation with FRAP and it was the only component where A549 and MDA-MB-231 cell lines have a positive correlation, 0.185 and 0.075, respectively.

As shown in Fig. 1 for grapevine leaves, the cultivars could be divided into three groups based on positions in the scores scatter plot of PCA. Group 1, includes the following cultivars Arvino, Magliocco dolce and Nocera. This group was characterized by higher contents of DPPH, TPC, ABTS test, FRAP test, β-carotene bleaching test at 30 and 60 min of incubation, TCA, resveratrol, caffeic acid and syringic acid. Component 2 includes the Magliocco canino and Gaglioppo cultivars, characterized by higher contents of gallic acid, rutin and myricetin and component 3, which includes Greco nero cultivar, characterized by

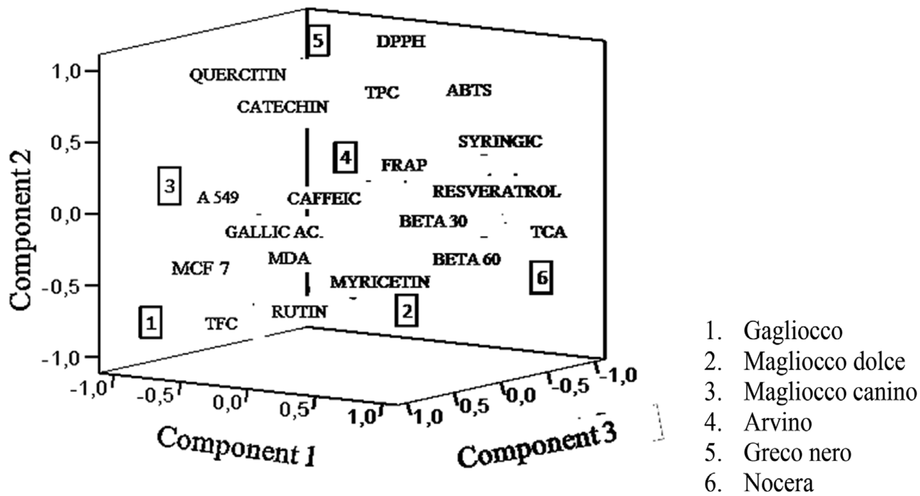

Fig. 1. Principal Component Analysis (PCA) of six grapevine leaves. Loadings plot and Scores scatter plot. The first three components of the PCA show 85% of the total variance: 48.55% for component 1, 21.15% for component 2 and 15.29% for component 3.

higher contents DPPH, TPC, ABTS, FRAP, catechin, quercetin. The results obtained from the PCA analysis showed the existence of chemical variability among samples obtained from leaves.

4. Conclusions

Results presented in this study demonstrated that Gaglioppo and Magliocco Dolce leaves extracts have shown to increase the defences against an excessive production of free radicals and exert a promising anti-proliferative activity against human Caucasian breast adenocarcinoma. These extracts are rich in bioactive compounds, mainly phenols that are known for their healthy properties. For this reasons their use in nutraceuticals or as ingredients in functional foods may support sustainable agricultural production and offer a new opportunity for by-products reutilization.

Conflicts of interest

The authors declare no conflicts of interest.

Abbreviations used

ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
BETA 30	β-carotene bleaching test at 30 min incubation; BETA 60, β-carotene bleaching test at 60 min incubation
BHT	Butylated hydroxytoluene
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Ability Power
GAS	Global Antioxidant Score
HPLC	High Performance Liquid Chromatography
RACI	Relative Antioxidant Capacity Index
SRB	sulforodamine B
TPC	total phenolic
TFC	total flavonoid
TCA	total carotenoid

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.007.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.03.007.

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