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Grape Stem Extracts From Three Native Greek Vine Varieties Exhibit Strong Antioxidant and Antimutagenic Properties

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Abstract. *Background/Aim: The winemaking procedure results in the generation of stems, a by-product that is harmful to the environment. Concomitantly, stems are rich in polyphenols and, hence, they are putatively beneficial for human health. Materials and Methods: In this study, the grape stem extracts derived from three native Greek vine varieties, namely Mavrodaphne, Muscat and Rhoditis were examined for their chemical composition and antioxidant and antimutagenic properties using a battery of in vitro biomarkers. Results: All extracts are rich in polyphenols. Moreover, they exhibit potent antioxidant and antimutagenic properties with the extract of Mavrodaphne being the strongest in reducing the DPPH$^{\bullet}$ and $O_2^{-\bullet}$ radicals and the Fe^{3+} and in protecting plasmid DNA from peroxyl radical-induced oxidative modification. Conclusion: Therefore, although they are serious pollutants, grape stems contain phytochemicals with important biological properties and can be used as (ingredients of) bio-functional foods to improve certain aspects of human health.*

Grape is the fruit of the grapevine that comprises approximately 80 species of vining plants (1). *Vitis vinifera* is a common grapevine belonging to the family Vitaceae and is native in the Mediterranean basin, central Europe, and south-western Asia (1). According to the archaeological evidence, grape is cultivated as a domesticated plant since 4,000-6,000 B.C. in the region of south Caucasus starting from the *Vitis vinifera* subsp. sylvestris, which is the progenitor of today's *Vitis vinifera* subsp. Vinifera (2). Vine is one of the oldest plants cultivated by humans that is also mentioned in the Bible, thus being an important piece of human culture (3). There are numerous grapevine varieties and on this basis, grape is typically consumed as a table fruit in the form of dried raisins or it is crushed to produce juice and wine. Grapes are a nutritional source of minerals such as phosphorus and calcium and vitamin A whereas they contain sugars (especially glucose and fructose) in amounts that depend on the variety (1). Apart from the above-mentioned constituents, grapes contain high amounts of polyphenolic compounds with essential roles in human health (4). It is established that grape polyphenols and especially resveratrol that is the most abundant, possess significant antioxidant, anti-diabetic and antimicrobial properties (5). Additionally, wine, the most common product of grapes is a fundamental pillar in the mediterranean diet (MD). Interestingly, Ancel Keys observed that the poor populations in the Italian countryside were healthier than their wealthy counterparts residing in New York. Seeking an explanation, he hypothesized that the nutritional habits and the way of life were related to the lower frequency of individuals suffering from cardiovascular disease (6). Therefore, he conducted the well-known "Seven Countries Study" (*i.e.*, Greece, Finland, Italy, Holland, United States, Yugoslavia, Japan) and concluded that a nutritional profile resembling to the MD (*i.e.*, consumption of fruit and vegetables, plenty of olive oil, confined animal fat and one or two glasses of red wine) was the explanation he quested (7).

Although small quantities of wine are an important constituent of MD, the chemical procedure for its production leads to the release of compounds with serious polluting

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burden to the environment. Indeed, grape stems that are by-products of the vinification process include the remains of grapevine such as leaves and tendrils (8). Grape stems are the less valorised residues that are generated after vinification since, in contrast to grape pomace that can be used as ingredients of animal feed in order to improve meat quality and redox status, they have practically fewer uses (9). This is why they are easily discarded in the environment leading to the pollution of soil and waters. It is worth mentioning that grape stems are rich in phytochemical compounds with important biological actions, namely polyphenols and mainly resveratrol, catechin, procyanidin B3 and gallic acid and, interestingly, they contain the aforementioned compounds in higher concentrations compared to the intact grape or wine (10). Nevertheless, the high amounts of polyphenols present in the stems may hamper their biodegradation and, hence, promote their accumulation in the environment (11). At the same time, the presence of polyphenolic compounds in grape stems gives them exceptional biological value (12). It has been shown that extracts derived from grape stems possess potent antioxidant activity *in vitro* (13) and in cell lines (14), whereas their anticarcinogenic and antiangiogenic roles have also been reported (15-17).

Although grape stems possess beneficial properties for human health, the studies on the biological roles of stems and especially of those generated from Greek grape varieties are scarce. Therefore, the main objective of the present study was to investigate the potential antioxidant and antimutagenic properties of extracts derived from stems of three native Greek vine varieties with special characteristics, namely Mavrodaphne, Muscat and Rhoditis. The ultimate goal is to holistically characterize these plant extracts in terms of their biological roles *in vitro* and *in vivo* in order to putatively use them as (ingredients of) bio-functional foods (18, 19). Regarding the three studied grape varieties, Mavrodaphne whose name literally means black laurel is mainly cultivated in Patras, Peloponnese, and is a highly identifiable Greek variety. The Mavrodaphne grape has a near-black colour and the produced wine has an aroma of dried prunes and currants and resembles to the Port dessert wines (20). Muscat (or Moscato) is a prestigious white grape variety comprising many grape families and constitutes a native Greek product. It is stated that the cultivation of Muscat grapes started in Greece and that this variety set the foundations of wine making procedure worldwide (21). In this study, we have used an extract originated from a Muscat grape variety that is a product of the city of Patras. Finally, Rhoditis is a pink-skinned grape variety, which is also cultivated in Patras's vineyards. It is, however, widely planted throughout Greece and it is used for the generation of retsina, a wine product that is a trademark of Greece (22). The three aforementioned grape varieties are used for the production of protected designation of origin (PDO) wines.

Materials and Methods

Collection of the plant material. The tested samples were collected from Patras vineyards in 2018. Samples of grape stems were manually collected from three vine varieties, specifically Mavrodaphne, Muscat and Rhoditis. After collection, the stems were air-dried, powdered using a mill and stored at RT.

Extraction process. Fifty grams of the stem samples were dissolved into a 200 ml of a mixture of methanol (MeOH)/H_2O/1.0 N HCl (90:9.5:0.5 v/v) and were sonicated for 10 min in an ultrasonic bath (Bandelin Sonorex super, model RK100SH, Berlin, Germany). The solvent was separated by filtration, and the remaining solid was re-extracted two additional times, using the same solvent system and procedure. The extracts were evaporated under vacuum forming a slurry, which was dissolved in 30 ml of MeOH/H_2O (1:1) and centrifuged (5,000 g, 10 min, 25˚C). Afterwards, the supernatant liquid was extracted with petroleum ether (3×30 ml) to remove the lipids and concentrated under vacuum. The remaining residue was poured into 30 ml of brine and extracted repetitively with ethyl acetate (EtOAc, 4×30 ml). As a result, all sugars remained in the aqueous layer. Then, the combined organic layers were dried over anhydrous magnesium sulfate and evaporated under vacuum. The remaining solid was weighed and dissolved in MeOH (1 mg/ml), membrane filtered (0.45 μm) and subjected to liquid chromatography analysis. To avoid polyphenol degradation, the aforementioned procedure was performed in the absence of direct sunlight and at temperatures ranging between 30 and 35˚C.

Determination of the extracts chemical Composition by HPLC and HPLC-ESI/MS. The chemical composition of the extracts was determined using HPLC analysis that was performed on a Hewlett Packard HP1100 (Hewlett Packard, Palo Alto, CA, USA) equipped with an Agilent 1100 diode-array detector (Agilent Technologies, Santa Clara, CA, USA) (measuring absorbance over the full wavelength range during the entire run), a quaternary pump, degasser and coupled to HP ChemStation utilizing the manufacturer's 5.01 software package system. A Lichosphere C18 chromatographic column obtained from Merck, Darmstadt, Germany (250 mm × 4.1 mm, particle size 5 μm) was used and connected with a guard column of the same material (8×4 mm). Injection was by means of a Rheodyne injection valve (model 7725I) with a 20 μl fixed loop obtained from Merck, Darmstadt, Germany. For the chromatographic analyses HPLC-grade H_2O was prepared using a Milli-Q system (Merck Millipore, Burlington, MA, USA), whereas all HPLC solvents (except acetonitrile) were filtered through cellulose acetate membranes of 0.45 μm pore size prior to use. The mobile phase was composed of a gradient system of 0.3% acetic acid in water (A) and acetonitrile (B). The flow rate was maintained at 1 ml/min and the column gradient elution program consisted of 25% B (0 min). 25% B (5 min). 30% B (10 min). 40% B (15 min), 50% B (20 min), 70% B (30 min) where it remained for additional 5 min, and returned during 2 min to initial conditions, where it stayed for additional 2 min. This routine was followed by a 15-min equilibration period with the zero-time mixture prior to injection of the next sample. Peaks were identified by comparing their retention times and UV–vis spectra with the reference compounds and data were quantitated using the corresponding curves of the reference compounds as standards.

Confirmatory UPLC-MS/MS analysis was carried out on a Thermo Scientific Ultra High Performance Liquid Chromatography

system coupled to a TSQ Quantum Vantage (Thermo Fischer Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer. Mass spectrometric analysis was conducted using a heated electrospray ionization (HESI) operating in two complementary modes (positive and negative mode). Selected ion monitoring (SIM) mode was primarily used to confirm the presence of analytes. In selected cases of compounds tandem mass spectrometry (MS/MS) utilizing the multiple reaction monitoring mode (MRM) was employed for additional confirmation. The working conditions were the following: spray voltage 4.2 kV, vaporizer and capillary temperatures 280 and 260˚C, respectively, while sheath and auxiliary gas at 60 and 40 arbitrary units, correspondingly. The LC separation was achieved on a Hypersil Gold. 3 μm. 150×3 mm i.d. chromatographic column (Thermo Fischer Scientific, San Jose, CA, USA). The mobile phase and the gradient system were identical to the abovementioned for the HPLC-UV analysis, using a flow rate of 0.3 ml/min.

Determination of the total polyphenolic content (TPC) of the extracts. For the determination of the total polyphenolic content, 20 μl of each extract was mixed with 1.6 ml of dH_2O, and 100 μl of Folin-Ciocalteu reagent (0.2 N) as previously described (23, 24). Then, 280 μl of Na_2CO_3 solution (200 g/l) was added and after 1 h incubation in the dark 25˚C, the optical density was monitored at 765 nm. TPC was calculated on the basis of a calibration curve of gallic acid (concentration range: 50-250 mg/l, R^2=0.997). The results are expressed as gallic acid equivalents (GAEs) using the standard curve (absorbance *versus* concentration) prepared from solutions with known gallic acid concentrations.

Determination of total flavonoid content (TFC) of the extracts. The total flavonoid content of the extracts was determined using a modified colorimetric method (25). In particular, 1 ml of each methanolic extract was added into a 10 ml flask containing 4 ml of dH2O. Then, 300 μl of 5% NaNO2 was added and the mixture was allowed to stand for 5 min at 25˚C. Then, 300 μl of 10% AlCl3*6H2O was added, the mixture was allowed to stand for 1 min at 25˚C and 2 ml of 1 M NaOH was added. The solution was diluted to a final volume equal to 10 ml by adding dH_2O and the absorbance of the solution *versus* the blank was monitored at 510 nm. The results are expressed as catechin equivalents using a standard curve (absorbance *versus* concentration) prepared from catechin samples with known concentrations.

The assay for the determination of the capacity of the extracts to reduce DPPH• radical. The ability of the extracts to scavenge DPPH• radical was measured according to a protocol previously described (19, 26). Briefly, 1 ml of freshly made methanolic solution of DPPH• radical (100 μM) was mixed with the tested extracts dissolved in dH_2O at a wide range of concentrations (0.75-37.5 μg/ml). The absorbance of the samples was monitored at 517 nm after a 20 min incubation in dark at 25˚C. In each experiment, the tested sample alone in methanol was used as the blank and DPPH• alone in methanol was used as the control. The percentage of radical scavenging capacity (RSC) of the tested extracts was calculated according to the following equation:

$$\%\ \text{DPPH}^\bullet \text{ radical scavenging activity} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100,$$

where $Abs_{control}$ and Abs_{sample} are the absorbance values of the control and the tested sample, respectively. Moreover, in order to compare the RCS of the extracts, the IC_{50} value showing the concentration that induced the scavenging of the DPPH• radical at 50% was estimated.

The assay for the determination of the capacity of the extracts to reduce ABTS•+ radical. The ABTS•+ radical scavenging ability of the tested extracts was determined as previously described (19, 26). Briefly, the reaction was carried out in 1 ml and the mixture contained ABTS•+ (1 mM), hydrogen peroxide (H_2O_2, 30 μM) and horseradish peroxidase (HRP) (6 μM). The samples were vigorously mixed after incubation in the dark at 25˚C for 45 min. Subsequently, 10 μl of each extract at a wide range of concentrations (0.75-37.5 μg/ml) were added to the reaction mixture and the absorbance at 730 nm was monitored. In each experiment, the HRP free tested sample was used as the blank and the ABTS•+ radical solution with H_2O and HRP was used as the control. The percentage of RSC and the IC_{50} values of the extracts were determined similarly to the DPPH• assay.

The assay for the determination of the capacity of the extracts to reduce Fe^{3+} to Fe^{2+} (i.e., the reducing power assay). The reducing power of the extracts was determined as previously described (19, 24). In brief, the extracts were dissolved in phosphate buffer (0.2 M, pH=6.6) at a wide range of concentrations (0.375-15 μg/ml). At first, 250 μl of each sample solution was added to 250 μl of potassium ferricyanide (1% w/v) and incubated at 50˚C for 20 min. The samples were cooled on ice for 3 min. Subsequently, 250 μl of TCA (10%) was added in the mixture and the samples were centrifuged (5,000 g, 10 min, 25˚C). Then, 250 μl of dH_2O and 500 μl of ferric chloride (0.1% w/v) were added to 600 μl of the supernatant, the samples were incubated in the dark at 25˚C for 10 min and the absorbance was monitored at 700 nm. In order to compare the reducing power of each extract the RP0.5AU value which shows the extract concentration which causes absorbance of 0.5 AU at 700 nm was calculated. The reducing power of the extracts is also expressed as IC_{50}.

The assay for the determination of the capacity of the extracts to reduce superoxide radical ($O_2^{\bullet-}$). The superoxide radical scavenging capacity of the extracts was evaluated as previously described (19, 24). Briefly, 50 ml of each extract was dissolved in dH_2O at a wide range of concentrations (5-50 μg/ml) and was added in 625 μl of Tris-HCl (16 mM, pH=8), 125 μl of NBT (300 μM) and 125 μl of NADH (60 μM). Then, the samples were incubated in the dark at 25˚C for 5 min and the absorbance was monitored at 560 nm. The percentage of RSC and the IC_{50} values were determined similarly to the DPPH• assay.

The assay for the determination of the capacity of the extracts to reduce hydroxyl radical (OH•). This ability of the extracts to reduce OH• was determined as previously described (19, 24). In brief, 75 μl of each extract dissolved in dH_2O at a wide range of concentrations (2-100 μg/ml) were added in 225 μl of phosphate buffer (0.2 M, pH=7.4), 75 μl of 2-deoxyribose (10 mM), 75 μl of $FeSO_4$-EDTA (10 mM), 30 μl of H_2O_2 (10 mM) and 270 μl of dH_2O. The samples were incubated in the dark at 37˚C for 1 h. Subsequently, 375 μl of trichloroacetic acid (TCA, 2.8%) and 375 μl of 2-thiobarbituric acid (1% w/v) were added and the samples were incubated at 95˚C for 10 min. Then the samples were cooled on ice for 3 min, centrifuged

(5,000 g, 10 min, 25˚C) and the absorbance was monitored at 520 nm. The samples without H_2O_2 were used as the blank and the samples without protein were used as the control. The percentage of RSC and the IC_{50} values were determined similarly to the DPPH• assay.

The assay for the determination of the capacity of the extracts to protect plasmid DNA strand scissions induced by peroxyl radical (ROO•). The assay was performed as described previously (19, 27). The protective activity of the tested extracts against ROO• was based on the inhibition of the conversion of the normal supercoiled form of DNA to the open circular, which is an indication of oxidative modification. The reaction mixture (10 μl) containing Bluescript-SK+ plasmid DNA (1 μg), various concentrations of the tested extracts (2-230 μg/ml) and 2,2'-azobis (2-amidinopropane hydrochloride (AAPH, 570 mM) in PBS was incubated for 45 min at 37˚C. The reaction was terminated by the addition of loading buffer (3 μl, 0.25% bromophenol blue and 30% glycerol) and analyzed in 0.8% agarose gel electrophoresis at 70 V for 1 h. The gels were stained with ethidium bromide (0.5 μg/ml), distained with dH2O, photographed by UV transillumination using the Vilber Lourmat photodocumentation system (DP-001.FDC, Torcy, France) and analyzed with the Gel-Pro Analyzer version 3.0 (Media Cybernetics, Silver Spring, Rockville, MD, USA).

The percentage inhibition was calculated using the following equation:

$$\% \text{ inhibition} = [(S - So)/(S_{control} - So)] \times 100,$$

where $S_{control}$ is the percentage of the supercoiled DNA of the negative control sample (plasmid DNA alone), *So* is the percentage of the super-coiled plasmid DNA of the positive control sample (without the tested extracts but in the presence of the radical initiating factor) and S is the percentage of the supercoiled plasmid DNA of the sample with the tested extracts and the radical initiating factor. The IC_{50} values were determined similarly to the DPPH• assay.

The assay for the determination of the capacity of the extracts to inhibit the mutations in Salmonella typhimurium (i.e., the Ames test). The bacterial strain that was used in order to determine the antimutagenic activity of the extracts was *Salmonella typhimurium* TA102 and the protocol was based on Maron and Ames (1983) (28), as previously described (19). For each experiment a frozen stock culture stored at –80˚C was thawed at 25˚C. Then, 700 μl of the stock culture were used to inoculate 30 ml of autoclaved Oxoid nutrient broth no. 2. The inoculated culture was incubated in the dark at 37˚C for 2.5 h until the cells reached a density of 1-2×10^9 colony forming units (CFU/ml, OD_{540} between 0.1 and 0.2AU). When the strain was in the exponential phase, the experiment began. The following substances were added in plastic falcon tubes at 45˚C±2˚C: 2 ml of top agar, 50 μl of various concentrations of each extract, 50 μl of *tert*-butylhydroperoxide (*t*-BOOH) solution (0.4 mM final concentration) and 100 μl of the bacterial culture. The contents of the tubes were mixed vigorously and poured onto the surface of glucose minimal agar plates. When the top agar was hardened the plates were inverted and placed in an incubator at 37˚C for 48 h. Afterwards, the histidine revertant colonies (His^+) were counted. Before counting, the agar plates were microscopically checked for toxicity. Each assay included both positive (the oxidizing agent alone) and negative controls (plates without the oxidizing agent or the tested extract). Also, each antioxidant was examined at the two highest concentrations of the extracts used for possible induction of mutations. The number of induced revertants was obtained by subtracting the number of the spontaneous revertants from the number of the revertants on the plates containing the mutagen and/or the antioxidant. The percentage inhibition of mutagenicity was calculated as follows:

Inhibition=[1-number of colonies/plate with oxidant+ test compound//number of colonies/plate with oxidant alone] ×100.

Statistical analysis. The data were analyzed with one-way ANOVA followed by Tukey's test using the statistical package for social sciences (SPSS, Inc., Chicago, IL, USA, version 21.0). All experiments were carried out in triplicate and on at least two separate occasions. All results are expressed as mean±SD (*i.e.*, standard deviation). The level of the statistical significance was set at $p<0.05$.

Table I. *The chemical composition of the three tested grape stem extracts.*

Polyphenolic compound	Concentration (μg/mg of extract)		
	Mavrodaphne	Muscat	Rhoditis
Gallic acid	5.581	4.571	3.110
Gallocatechin	0.037	0.089	0.047
Procyanidin B1	8.907	3.607	10.010
Catechin	1.913	1.860	3.602
Procyanidin B2	1.454	1.194	2.999
2,5 Dihydroxybenzoic acid	0.259	0.084	0.332
Epicatechin	0.844	0.766	1.678
Caffeic acid	3.700	1.658	2.048
Rutin	0.028	0.096	0.287
Polydatin	0.099	0.131	0.129
Quercitrin-3-b-glucoside	0.340	0.199	0.761
Ellagic acid	0.376	0.466	0.933
Hesperidin	0.050	0.058	0.035
Quercitrin	0.152	0.072	0.121
Trans-Resveratrol	0.450	0.402	0.470
Quercetin	0.620	0.359	0.469
TPC	374.765	264.795	359.865
TFC	14.047	17.478	12.630

TPC: Total polyphenolic content expressed as mg of gallic acid equivalent/g dried weight of the extract; TFC: total flavonoid content expressed as mg of catechin equivalent/g dried weight of the extract.

Results

Chemical composition of the grape stem extracts. The chemical composition of the three tested extracts is depicted in Table I. The stem extract derived from Mavrodaphne is rich in gallic acid and caffeic acid, a hydroxybenzoic and a hydroxycinnamic acid, respectively, whereas it contains higher amounts of the flavonol quercetin and quercitrin, a flavonol glycoside, compared to the other two extracts. The extract generated from Muscat contains higher concentrations of gallocatechin, a flavanol, polydatin, which is a stilbene and hesperidin, a flavanon glycoside, in

Table II. *The IC_{50} values of the three tested grape stem extracts in the biomarkers/bioassays that were used to evaluate their antioxidant and antimutagenic properties.*

Grape stem extract	Biomarker/Bioassay	IC_{50} (μg of extract/ml±SD)
Mavrodafne	$DPPH^{\bullet}$	10.88±1.5
	$ABTS^{\bullet+}$	8.07±0.19
	$OH^{\bullet}$	166.62±12.16
	$O_2^{-\bullet}$	36.12±1.48
	Reducing Power	2.59±0.48
	Plasmid relaxation assay ($ROO^{\bullet}$)	131.38±7.2
	Ames test	4.66±0.45
Muscat	$DPPH^{\bullet}$	15.61±0.38*
	$ABTS^{\bullet+}$	10.26±0.66
	$OH^{\bullet}$	220.02±17.9
	$O_2^{-\bullet}$	44.63±3.07*
	Reducing power	3.37±0.3*
	Plasmid relaxation assay ($ROO^{\bullet}$)	151.39±0.2
	Ames test	3.94±0.17
Rhoditis	$DPPH^{\bullet}$	14.87±1.05*
	$ABTS^{\bullet+}$	11.52±0.77*
	$OH^{\bullet}$	106.32±21.29$^{\#}$
	$O_2^{-\bullet}$	43.76±0.71
	Reducing Power	2.77±0.13
	Plasmid relaxation assay ($ROO^{\bullet}$)	168.06±8*
	Ames test	4.58±0.004

SD: Standard deviation; *Statistically significant compared to Mavrodaphne for the same biomarker/bioassay. $^{\#}$Statistically significant compared to Muscat for the same biomarker/bioassay.

comparison to Mavrodaphne and Rhoditis. Finally, the chemical compounds detected in the extract of Rhoditis in higher amounts compared to Mavrodaphne and Muscat are two members of proantho-cyanidins (procyanidin B1 and B2), the flavanols catechin and epicatechin, two hydroxybenzoic acids (2,5 dihydroxybenzoic acid, ellagic acid), the flavonol rutin, quercitrin-3-b-glucoside which is a flavonol glycoside and trans-resveratrol, a well-known stilbene.

Total polyphenolic and flavonoid content of the extracts. The total polyphenolic content (TPC) of the Mavrodaphne stem extract (*i.e.*, 374.765 mg of gallic acid equivalent/g dried weight of the extract) was higher than the TPC of the Muscat and Rhoditis extracts (*i.e.*, 264.795 and 359.865 mg of gallic acid equivalent/g dried weight of the extract, respectively). With respect to the total flavonoid content, the Muscat extract was richer (*i.e.*, 17.478 mg of catechin equivalent/g dried weight of the extract) compared to the extracts of Mavrodaphne and Rhoditis (*i.e.*, 14.047 and 12.630 mg of catechin equivalent/g dried weight of the extract, respectively).

Antioxidant properties of the extracts. The obtained results indicate that the stem extract of Mavrodaphne is the most potent in terms of the antioxidant and antimutagenic properties (Table II). Specifically, it exhibits strong antioxidant activity since it has the ability to reduce $DPPH^{\bullet}$ radical in lower IC_{50} (*i.e.*, 10.88 μg of extract/ml) compared to the extracts from Muscat and Rhoditis (IC_{50} values equal to 15.61 and 14.87 μg of extract/ml, respectively). Mavrodaphne is also more efficient in reducing superoxide radical ($O_2^{-\bullet}$) in comparison to Muscat (IC_{50} values equal to 36.12 and 44.63 μg of extract/ml, respectively). Additionally, Mavrodaphne is more potent in reducing Fe^{+3} to Fe^{+2} assessed through the reducing power assay (IC_{50} value: 2.59 μg of extract/ml) compared to Muscat and Rhoditis (IC_{50} values equal to 3.37 and 2.77 μg of extract/ml, respectively).

Antimutagenic properties of the extracts. The stem extract of Mavrodaphne had also the ability to protect plasmid DNA from the mutagenic activity of peroxyl radicals ($ROO^{\bullet}$), evaluated by the plasmid relaxation assay, in lower concentration than the Rhoditis extract (IC_{50} values equal to 131.38 and 168.06 μg of extract/ml, respectively). Overall, the grape stem extract derived from Mavrodaphne vine exerted statistically significant stronger antioxidant and antimutagenic properties compared to the extracts of Muscat and Rhoditis (Table II).

Discussion

The present study investigated the biological properties of three grape stem extracts derived from Mavrodaphne, Muscat and Rhoditis, which are native Greek vine varieties.

All tested extracts exhibited strong antioxidant and antimutagenic activity. Overall, the extract generated from Mavrodaphne is the most potent compared to the other two since it is more efficient in reducing the DPPH$^{\bullet}$ and the $O_2^{-\bullet}$ radicals and the Fe^{3+} to Fe^{2+} assessed through the reducing power assay. In addition, it appears that it protects the peroxyl radical induced oxidative modification of plasmid DNA in lower concentration than the extracts from Muscat and Rhoditis (Table II). These findings assert that all three grape stem extracts are important antioxidant agents and, hence, potentially beneficial for human health. Therefore, they may be used as constituents of bio-functional foods.

On the basis of the chemical composition of the tested extracts, it is evident that they are rich in polyphenolic compounds that belong to the greater class of flavonoids and specifically, flavanols and flavonols along with proanthocyanidins, hydroxybenzoic acids and stilbenes. However, the comparison of the constituents between the three extracts reveals no distinct differentiations. This means that neither of the extracts surpasses the others in terms of the concentration of specific polyphenols. It has to be mentioned, though, that the Mavrodaphne extract has the higher total polyphenolic content than the other two (Table I). Hence, the fact that it exhibits the most potent antioxidant and antimutagenic properties could partly attributed to its richness in polyphenols. It is also interesting that the IC_{50} values of all extracts in the reducing power assay is approximately 50-fold lower than those in the plasmid relaxation assay. This is in line with experimental evidence reporting that flavonoids exert greater ability to protect against metal-ion (Fe ions in our case) induced oxidation than peroxidation due to peroxyl radicals (29).

A crucial innovation of the present investigation is the evaluation of the notable biological properties of grape stems in the form of extracts that are a rather underestimated source of chemical compounds (*i.e.*, polyphenols) with beneficial roles in human health (10). Moreover, we studied the extracts derived from three native Greek vine varieties that are among the most distinguishable and, at the same time, the less examined for their putative antioxidant properties. The manifold roles of polyphenolic compounds in human health have been demonstrated by numerous studies. Indeed, there is evidence implying that they contribute to the prevention or inhibition of cardiovascular diseases (30) and specific cancer types (31), whereas they delay the degenerative effects of aging, which is a redox related condition (4). Furthermore, muscadine grape extracts rich in polyphenols have shown strong positive correlation with growth inhibition of breast cancer cells (32). Polyphenolic compounds are also antimicrobial and anti-inflammatory agents especially due to their multifaceted antioxidant properties, which are ascribed either by the direct scavenging of free radicals and chelation of metal ions or through the regulation of redox signaling pathways and the expression of genes coding for enzymes involved in the antioxidant defence of blood and tissues (18, 33).

Apart from the direct advantageous effects of polyphenols on human health, they can also indirectly maintain a healthy wellbeing through their usage as food preservatives (34). Due to their antioxidant properties they are able to preserve the colour, the flavour and the odour of foods and their nutritional value by blocking the oxidative spoiling of vitamins, lipids and other fundamental constituents (35, 36). Moreover, they protect foods against microbes and therefore they are considered as promising bio-functional foods (37). The bioavailability of polyphenolic compounds is also another trait that leads to their wide usage (38). To that end, it has repeatedly demonstrated that procyanidins and flavonoids derived from grapes are rapidly absorbed in plasma where their peak concentration is observed 2-3 h after consumption (39, 40). Moreover, specific metabolites of polyphenols after a two-week consumption of red wine were detected in plasma indicating that they are normally absorbed by human blood and, hence, they can act beneficially (4, 30). Nevertheless, we have to keep in mind that polyphenols in higher concentrations can also act as pro-oxidants (4). Additionally, the effects of some high-molecular weight polyphenolic compounds may be negative because they cannot be absorbed and phytochemicals with specific molecular structures usually have the same impact (41).

The polyphenolic compounds are the constituents with the higher concentration in the tested grape stem extracts. Although grape pomace and seeds have been extensively studied, stems have surprisingly gained less attention regarding their biological roles (42). Grape stems are collected before the winemaking procedure and constitute 5% of the wine by-products. They are usually incorporated in ruminant feed or for the generation of compost, but in the majority of the cases they are disposed to the environment (43). Their disposal causes serious environmental problems because stems contain high organic material that is biodegraded in the soil and waters (11). Therefore, one of the greater challenges for the scientific community is to exploit this by-product so that the human health is promoted and the environment is protected. This could happen through the recovery and isolation of the phytochemicals that are contained in the grape stems. These compounds can subsequently be used in favour of human society as it has been stated in the previous paragraphs due to their potent antioxidant properties (13-17). Another way is to utilize the grape stems per se by incorporating them into the cosmetics, and in products of pharmaceutical and food industry (34). In that sense, they can be incorporated in edible products in order to promote certain aspects of human health and, thus, can be adopted as bio-functional foods (42). Apart from the obvious advantages, the stems can offer economic profit also since they are considered a cheap source of antioxidants, which can easily be recovered (10).

In conclusion, the present study reports that the grape stems derived from Mavrodaphne, Muscat and Rhoditis native Greek vine varieties are, to our knowledge, evaluated for the first time in terms of their antioxidant and antimutagenic properties. All of them exert strong biological activities and, according to our findings, the extract of Mavrodaphne is the most potent compared to the other two. These extracts are rich sources of polyphenolic compounds to which their antioxidant activity is mainly attributed to. Given that the grape stems have scarcely been studied, their beneficial biological role, observed herein, dictates that they can be used as ingredients of bio-functional foods in order to promote or improve certain aspects of human health.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

DK, KP, SH: Conception and design of the study; EV, SH: Conduction of the experiments. ASV, EV: Statistical analysis; ASV, MK, EA, DK: Interpretation of the data; ASV, EV: Writing of the manuscript. All Authors drafted the final version of the manuscript.

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References

1 Bender DAA: Dictionary of food and nutrition. New York: Oxford University Press, 2009. DOI: 10.1093/acref/9780199234875.001.0001

2 McGovern PE: Ancient wine: The search for the origins of viniculture. Princeton Univ Press, Princeton, 2003.

3 Myles S, Boyko AR, Owens CL, Brown PJ, Grassi F, Aradhya MK, Prins B, Reynolds A, Chia JM, Ware D, Bustamante CD and Buckler ES: Genetic structure and domestication history of the grape. Proc Natl Acad Sci USA *108(9)*: 3530-3535, 2011. PMID: 21245334. DOI: 10.1073/pnas.1009363108

4 Xia EQ, Deng GF, Guo YJ and Li HB: Biological activities of polyphenols from grapes. Int J Mol Sci *11(2)*: 622-646, 2010. PMID: 20386657. DOI: 10.3390/ijms11020622

5 Latruffe N and Rifler JP: Bioactive polyphenols from grapes and wine emphasized with resveratrol. Current Pharmaceutical Design *19(34)*: 6053-6063, 2013. PMID: 23448444. DOI: 10.2174/1381612811319340002

6 Keys AB and Keys M: How to eat well and stay well, the Mediterranean way. New York: Doubleday, 1975.

7 Keys AB: Seven countries: a multivariate analysis of death and coronary heart disease. Cambridge, Mass Harvard University Press, 1980. DOI: 10.1001/jama.1981.03310300063026

8 Gonzalez-Centeno MR, Rossello C, Simal S, Garau MR, Lopez F and Femenia A: Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomaces and stems. LWT Food Sci Technol *43*: 1580e-1586e, 2010. DOI: 10.1016/j.lwt.2010.06.024

9 Kafantaris I, Kotsampasi B, Vladimiros C, Makri S, Stagos D, Gerasopoulos K, Petrotos K, Goulas P and Kouretas D: Effects of dietary grape pomace supplementation on performance, carcass traits and meat quality of lambs. In Vivo *32(4)*: 807-812, 2018. PMID: 29936462. DOI: 10.21873/invivo.112311

10 Barros A, Gironés-Vilaplana A, Texeira A, Baenas N and Domínguez-Perles R: Grape stems as a source of bioactive compounds: Application towards added-value commodities and significance for human health. Phytochem Rev *14*: 921-931, 2015. DOI: 10.1007/s11101-015-9421-5

11 Makris DP, Boskou G and Andrikopoulos N: Polyphenolic content and *in vitro* antioxidant characteristics of wine industry and other agri-food solid waste extracts. J Food Comp Anal *20*: 125-132, 2007. DOI: 10.1016/j.jfca.2006.04.010

12 Shrikhande A: Wine by-products with health benefits. Food Res Int *33(6)*: 469-474, 2000. PMID: 28454964. DOI: 10.1016/S0963-9969(00)00071-5

13 Spanou C, Veskoukis A.S, Stagos D, Liadaki K, Anastasiadi M, Haroutounian S.A, Tsouka M, Tzanakouli E and Kouretas D: Effects of grape extracts on the *in vitro* activity of enzymes involved in oxidative stress regulation. In Vivo *25(4)*: 657-662, 2011. PMID: 21709010.

14 Goutzourelas N, Stagos D, Spanidis Y, Liosi M, Apostolou A, Priftis A, Haroutounian S, Spandidos DA, Tsatsakis AM and Kouretas D: Polyphenolic composition of grape stem extracts affects antioxidant activity in endothelial and muscle cells. Mol Med Rep *12(4)*: 5846-5856, 2015. PMID: 26300227. DOI: 10.3892/mmr.2015.4216

15 Stagos D, Apostolou A, Poulios E, Kermeliotou E, Mpatzilioti A, Kreatsouli K, Koulocheri SD, Haroutounian SA and Kouretas D: Antiangiogenic potential of grape stem extract through inhibition of vascular endothelial growth factor expression. J. Physiol Pharmacol *65(6)*: 843-852, 2014. PMID: 25554988.

16 Sahpazidou D, Geromichalos G.D, Stagos D, Apostolou A, Haroutounian S, Tsatsakis AM, Tzanakakis GN, Hayes AW and Kouretas D: Anticarcinogenic activity of polyphenolic extracts from grape stems against breast, colon, renal and thyroid cancer cells. Toxicol Lett *230(2)*: 218-224, 2014. PMID: 24508987. DOI: 10.1016/j.toxlet.2014.01.042

17 Apostolou A, Stagos D, Galitsiou E, Spyrou A, Haroutounian SA, Portesis N, Trizoglou I, Wallace Hayes A, Tsatsakis AM and Kouretas D: Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of *Vitis vinifera* stem extracts. Food Chem Toxicol *61*: 60-68, 2013. PMID: 23380202. DOI: 10.1016/j.fct.2013.01.029

18 Veskoukis AS, Tsatsakis AM and Kouretas D: Dietary oxidative stress and antioxidant defence with an emphasis on plant extract administration. Cell Stress Chaperones *17(1)*: 11-21, 2012a. PMID: 21956695. DOI:10.1007/s12192-011-0293-3

19 Veskoukis A, Kerasioti E, Priftis A, Kouka P, Spanidis Y, Makri S and Kouretas D: A battery of translational biomarkers for the assessment of the *in vitro* and *in vivo* antioxidant action of plant polyphenolic compounds: The biomarker issue. Curr Opin Toxicol *13*: 99-109, 2019. DOI: 10.1016/j.cotox.2018.10.001

20 Grossman HJ: Grossman's Guide to wines, spirits & beers. 7th Edition, Wiley, 1983.
21 Clarke O: Encyclopedia of Grapes, edition no. 1.; Websters International Publishers, Orlando, Florida, 2001.
22 Robinson J: "The Oxford Companion to Wine" Third Edition, Oxford University Press, 2006.
23 Singleton VL, Orthofer R and Lamuela-Raventos RM: Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Meth Enzymol *299*: 152-178, 1999. DOI: 10.1016/S0076-6879(99)99017-1
24 Kerasioti E, Apostolou A, Kafantaris I, Chronis K, Kokka E, Dimitriadou C, Tzanetou EN, Priftis A, Koulocheri SD, Haroutounian SA, Kouretas D and Stagos D: Polyphenolic composition of Rosa canina, Rosa sempervivens and Pyrocantha coccinea extracts and assessment of their antioxidant activity in human endothelial cells. Antioxidants (Basel) *8(4)*: 92, 2019. PMID: 30959906. DOI: 10.3390/antiox8040092
25 Jia Z, Tang MC and Wu JM: The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem *64*: 555-559, 1999. DOI: 10.1016/S0308-8146(98)00102-2
26 Veskoukis A.S, Kyparos A, Nikolaidis M.G, Stagos D, Aligiannis N, Halabalaki M, Chronis K, Goutzourelas N, Skaltsounis L and Kouretas D: The antioxidant effects of a polyphenol-rich grape pomace extract *in vitro* do not correspond *in vivo* using exercise as an oxidant stimulus. Oxid Med Cell Longev *2012*: 185867, 2012. PMID: 22693650. DOI: 10.1155/2012/185867
27 Kouka P, Tekos F, Valta K, Mavros P, Veskoukis AS, Angelis A, Skaltsounis AL and Kouretas D: Olive tree blossom polyphenolic extracts exert antioxidant and antimutagenic activities *in vitro* and in various cell lines. Oncol Rep *42(6)*: 2814-2825, 2019. PMID: 31661143. DOI: 10.3892/or.2019.7386
28 Maron DM and Ames BN: Revised methods for the Salmonella mutagenicity test. Mutat Res *113*: 173-215, 1983. PMID: 6341825. DOI: 10.1016/0165-1161(83)90010-9
29 Arora A, Nair MG and Strasburg GM: Structure-activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. Free Radic Biol Med *24*: 1355-1363, 1998. PMID: 9641252. DOI: 10.1016/s0891-5849(97)00458-9
30 Tsanga C, Higginsa S, Duthiea GG, Duthiea SJ, Howiea M, Mullena W, Leana MEJ and Crozier A: The influence of moderate red wine consumption on antioxidant status and indices of oxidative stress associated with CHD in healthy volunteers. Br J Nutr *93*: 233–240, 2005. PMID: 15788107. DOI: 10.1079/bjn20041311
31 Jung K, Wallig M and Singletary K: Purple grape juice inhibits 7,12-dimethylbenz-[a]anthracene (DMBA)-induced rat mammary tumorigenesis and *in vivo* DMBA-DNA adduct formation. Cancer Lett *233*: 279-288, 2006. PMID: 15878797. DOI: 10.1016/j.canlet.2005.03.020
32 Mendonca P, Darwish AG, Tsolova V, El-Sharkawy I and Soliman KFA: The anticancer and antioxidant effects of muscadine grape extracts on racially different triple-negative breast cancer cells. Anticancer Res *39(8)*: 4043-4053, 2019. PMID: 31366486. DOI: 10.21873/anticanres.13560
33 Soobrattee MA, Neergheena VS, Luximon-Rammaa A, Aruomab OI and Bahoruna T: Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. Mut Res Fund Mol Mech Mutagen *579*: 200-213, 2005. DOI: 10.1016/j.mrfmmm.2005.03.023
34 Doshi P, Adsule P, Banerjee K and Oulkar D: Phenolic compounds, antioxidant activity and insulinotropic effect of extracts prepared from grape (*Vitis vinifera* L) byproducts. J Food Sci Technol *52(1)*: 181-190, 2015. PMID: 25593367. DOI: 10.1007/s13197-013-0991-1
35 Aliakbarian B, Dehghani F and Perego P: The effect of citric acid on the phenolic contents of olive oil. Food Chem *116(3)*: 617-623, 2009. DOI: 10.1016/j.foodchem.2009.02.077
36 Moure A, Cruz JM, Franco D, Dominguez JM, Sineiro J and Dominguez H: Natural antioxidants from residual sources. Food Chem *72(2)*: 145-171, 2001. DOI: 10.1016/S0308-8146(00)00223-5
37 Rodriguez-Vaquero MJ, Alberto MR and Manca-de-Nadra MC: Antibacterial effect of phenolic compounds from different wines. Food Control *18*: 93-101, 2007. DOI: 10.1016/j.foodcont.2005.08.010
38 Urpi-Sarda M, Monagas M, Khan N, Lamuela-Raventos RM, Santos-Buelga C, Sacanella E, Castell M, Permanyer J and Andres-Lacueva C: Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. Anal Bioanal Chem *394*: 1545-1556, 2009. PMID: 19333587. DOI: 10.1007/s00216-009-2676-1
39 Bell JRC, Donovan JL, Wong R, Waterhouse AL, German JB, Walzem RL and Kasim-Karakas SE: (+)-Catechin in human plasma after ingestion of a single serving of reconstituted red wine. Am J Clin Nutr *71*: 103-108, 2000. PMID: 10617953. DOI: 10.1093/ajcn/71.1.103
40 Cilla A, Gonzalez-Sarrias A, Tomas-Barberan FA, Espin JC and Barbera R: Availability of polyphenols in fruit beverages subjected to *in vitro* gastrointestinal digestion and their effects on proliferation, cell-cycle and apoptosis in human colon cancer Caco-2 cells. Food Chem *114*: 813-820, 2009. DOI: 10.1016/j.foodchem.2008.10.019
41 Ugartondo V, Mitjans M, Lozano C, Torres JL and Vinardell MP: Comparative study of the cytotoxicity induced by antioxidant epicatechin conjugates obtained from grape. J Agric Food Chem *54*: 6945-6950, 2006. PMID: 16939362. DOI: 10.1021/jf061356i
42 Ruiz-Moreno MJ, Raposo R, Cayuela JM, Zafrilla P, Piñeiro Z, Moreno-Rojas JM, Mulero J, Puertas B, Giron F, Guerrero RF and Cantos-Villar E: Valorization of grape stems. Ind Crop Prod *63*: 152-157, 2015. DOI: 10.1016/j.indcrop.2014.10.016
43 Anastasiadi M, Pratsinis H, Kletsas D, Skaltsounis AL and Haroutounian SA: Grape stem extracts: polyphenolic content and assessment of their *in vitro* antioxidant properties. Food Sci Technol *48*: 316-322, 2012. DOI: 10.1016/j.lwt.2012.04.006

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