Determination of the polyphenolic content and the antioxidant activities of four indigenous Greek red and white wine varieties

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Abstract. Greece has a long history in viticulture and vinification, cultivating indigenous and imported grape varietals for the production of fine wines. The dominant Greek red grape varieties are Xinomavro and Agiorgitiko, while the most recognizable Greek white grape varieties are Assyrtiko and Malagouzia. In the present study, the biological activities of monovarietal wines, derived from the aforementioned indigenous Greek grape varieties, were investigated by evaluating their polyphenolic content and antioxidant potency *in vitro*, using a well-established panel of cell-free assays. According to the results obtained, the red wines contained higher levels of polyphenolic compounds and exhibited more prominent antioxidant activities than the white wines. Among the different wine varieties, Xinomavro was the most potent, exhibiting a high concentration of polyphenols and notable antioxidant properties, whereas Malagouzia was the least efficacious. Finally, the correlation analysis between the total polyphenolic content and antioxidant capacity of the red and white wine varieties revealed a proportional correlation in almost all cases, indicating that the antioxidant activities strongly correlated with the phenolic content. Overall, the findings of the present study suggest that the monovarietal wines of these indigenous Greek grape varieties, and particularly Xinomavro, are highly bioactive, and their moderate consumption may be associated with health-promoting effects.

Introduction

Viticulture and vinification are agricultural activities with a significant socio-economic impact on a global scale (1). The European Union (EU) is the world-leading wine producer and consumer (2). According to the European Commission, the total average wine production in the EU for the financial year 2021-2022 was 152,932,000 hectoliters (3). Despite its alcohol content, a low-to-moderate wine consumption has been associated with beneficial health effects, due to its high abundance in bioactive phytochemicals (4,5). More specifically, previous studies have demonstrated that wine is a rich source of polyphenols, plant secondary metabolites that possess potent antioxidant (6), anti-inflammatory (7) and cardioprotective (8,9) properties.

The wine phenolic composition and content is dependent on several parameters and has a substantial impact on its organoleptic characteristics and health-promoting effects (10). The grapevine variety is a key determinant of the phenolic content, with red varieties containing higher levels of phenol substances than white varieties. In addition to the cultivar, the pedoclimatic conditions, such as soil composition, temperature, relative humidity, exposure to sunlight, rainfall and wind, contribute significantly to the grape phenolic composition (11). Furthermore, the vinification process constitutes a decisive factor in the wine phenolic content. During the red wine vinification process, the grape juice is fermented with all grape parts, including the skin and seeds, resulting in a higher polyphenol extraction in the final product (12). On the contrary, during the white wine vinification process, the grape skin is removed prior to grape juice fermentation, hence decreasing the phenolic concentration (13). Finally, the process of wine aging, particularly in oak barrels, has a considerable impact on the phenolic composition of red wines (12). To be more specific, phenolic compounds released from oak barrels enhance the phenolic content of the aged wine and contribute to color stability, thus protecting against oxidation (14,15). Additionally, during the red wine maturation and aging process, co-pigmentation reactions occur, favored by the presence of oxygen, leading to polymerization or formation of new, more complex and stable pigments (16,17).

Greece is one of the oldest wine-producing regions worldwide, with the first evidence of vinification dating back to the 3rd millennium B.C., during the Minoan civilization. The nutritional value and the health benefits of wine were widely recognized in Ancient Greece. Therefore, wine was an integral part of the daily regimen, also related to economic, social, religious and cultural aspects. Currently, Greece is one the first wine-producing territories in the EU, with 3.2% of the EU area occupied by vines (18). Of particular interest is that the Greek vineyard hosts ~200 indigenous grape cultivars of *Vitis vinifera L.*, 60 of which are extensively used in

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the winemaking process for the production of fine red and white wines (19).

The dominant Greek red wine varieties are Agiorgitiko and Xinomavro. Agiorgitiko is traditionally cultivated in the Nemea region, in the northeastern part of the Peloponnese, and contributes to the production of the protected designation of origin (PDO) Nemea (20). Xinomavro is cultivated in Northern Greece and contributes to the production of the PDO Naoussa (21). As regards the Greek white wine varieties, Assyrtiko and Malagouzia are definitely the most recognizable. Assyrtiko, a white wine variety considered one of the finest in the Mediterranean basin, originates from the volcanic Aegean island of Santorini and contributes to the production of PDO Santorini (22). Finally, Malagouzia, a white wine variety saved from near extinction in the recent past, is mainly cultivated in Central Greece and Macedonia (23).

Based on the aforementioned information, the aim of the present study was to determine the phenolic content and antioxidant capacity of four indigenous Greek wines, namely the red wine varieties Agiorgitiko and Xinomavro, as well as the white wine varieties, Assyrtiko and Malagouzia. Towards this purpose, a complete set of *in vitro* cell-free screening techniques was applied for assessing the antiradical, reducing and antigenotoxic properties of the wine varieties (24). The present study aims to improve the current knowledge as regards the bioactivity of these indigenous Greek grape varieties by assessing their bioactive compound content and antioxidant potency, thus enhancing their competitiveness and recognition abroad.

Materials and methods

Sample information and preparation. A total of 32 commercial wines, eight of each wine variety, produced from various regions across Greece and bottled in 750 ml wine bottles, were randomly selected and acquired from a local wine store in Larissa, Greece. The alcohol content of Agiorgitiko wines ranged from 13.5 to 15% v/v with an average of 14.1% v/v, that of Xinomavro wines from 12.5 to 14.5% v/v with an average of 13% v/v, that of Assyrtiko wines from 13.5 to 14.8% v/v with an average of 14.2% v/v, and that of Malagouzia wines from 12.5 to 13.8% v/v with an average of 13% v/v. Each bottle was opened and the wine was divided into aliquots and stored at 4˚C, until further analysis.

Determination of wine total phenolic content (TPC)

Folin-Ciocalteu assay. The TPC was evaluated using the Folin-Ciocalteu phenol reagent (FCR; Merck KGaA), as previously described by Singleton *et al* (25). In detail, 20 μl of each wine sample (dilution 1:2 in deionized water (dH_2O) for red wines, no dilution for white wines) was added to test tubes containing 1 ml dH_2O. Subsequently, 100 μl FCR were added and the mixture was incubated in the dark at room temperature (RT) for 3 min. Following incubation, 280 μl of 25% w/v sodium carbonate anhydrous (Na_2CO_3) solution (Honeywell Research Chemicals) and 600 μl dH_2O were sequentially added and the mixture was incubated for 1 h in the dark at RT. Following incubation, the optical density (OD) was monitored at 765 nm using a UV/Visible spectrophotometer (U-1500, Hitachi, Ltd.). For the determination of TPC, a standard curve was prepared using various concentrations (50-1,500 μg/ml) of gallic acid. The results were expressed as mg of gallic acid equivalents (GAE)/ml of wine sample.

Determination of wine antioxidant properties

2,2-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging assay. $DPPH^{\bullet}$ scavenging capacity was evaluated on the basis of the method described in the study by Brand-Williams *et al* (26). More specifically, 50 μl of each wine sample (0.25-8 μl/ml for red wines and 2.5-80 μl/ml for white wines) serially diluted in dH_2O was mixed with 900 μl of methanol (MeOH) and 50 μl of $DPPH^{\bullet}$ solution (2 mM; Alfa Aesar) in MeOH. In each experiment, a blank containing 1 ml MeOH and a negative control containing 950 μl MeOH and 50 μl $DPPH^{\bullet}$ solution in MeOH were prepared. Furthermore, vitamin C (Merck KGaA) was used as a positive control. The samples were vortexed vigorously and incubated in the dark at RT for 20 min. The OD was then measured at 517 nm using a UV/Visible spectrophotometer (U-1500, Hitachi, Ltd.). The radical scavenging capacity percentage (% RSC) was calculated using the following equation:

$$\%\,RSC = \frac{(ODcontrol - ODsample)}{ODcontrol} x\ 100$$

The half-maximal inhibitory concentration (IC50) was calculated from the linear regression curve by plotting the % RSC against the corresponding concentrations. The IC50 value represents the concentration of the wine sample required to neutralize the 50% of the corresponding free radicals. All analyses were carried out in triplicate and at least in two separate occasions.

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) scavenging assay. $ABTS^{\bullet+}$ scavenging capacity was evaluated on the basis of the method described in the study by Cano *et al* (27). More elaborately, 400 μl dH_2O, 500 μl ABTS solution (1 mM; Alfa Aesar) in dH_2O, 50 μl hydrogen peroxide (H_2O_2; 30 μM; Merck KGaA), and 50 μl horseradish peroxidase (HRP; 6 μM; SERVA Electrophoresis GmbH) were sequentially added to test tubes. A blank containing 450 μl dH_2O, 500 μl ABTS solution and 50 μl H_2O_2, as well as a negative control comprising 400 μl dH_2O, 500 μl ABTS solution, 50 μl H_2O_2 and 50 μl HRP were also prepared. Furthermore, vitamin C was used as a positive control. The samples were vortexed vigorously and incubated in the dark at RT for 45 min. Subsequently, 50 μl of each wine sample (0.3125-20 μl/ml for red and white wines) serially diluted in dH_2O was added, the samples were vortexed, and the OD was monitored at 730 nm using a UV/Visible spectrophotometer (U-1500, Hitachi, Ltds). The % RSC was calculated using the aforementioned equation. The IC50 value was calculated from the linear regression curve by plotting the % RSC against the corresponding concentrations. All analyses were carried out in triplicate and at least in two separate occasions.

Superoxide radical ($O_2^{\bullet-}$) scavenging assay. The $O_2^{\bullet-}$ scavenging capacity was evaluated based on the method described in the study by Gülçin *et al* (28). More specifically, 50 μl of each wine sample (0.25-8 μl/ml for red wines and 0.625-20 μl/ml for white wines) diluted in dH_2O was added to test tubes and mixed with 625 μl Tris-HCl buffer (16 mM,

pH 8.0), 125 μl nitro blue tetrazolium (NBT; 300 μM; SERVA Electrophoresis GmbH), 125 μl nicotinamide adenine dinucleotide (NADH; 468 μM; SERVA Electrophoresis GmbH) and 125 μl phenazine methosulfate (PMS; 60 μM; Merck KGaA). A blank containing 800 μl Tris-HCl buffer, 125 μl NBT and 125 μl NADH, as well as a negative control comprising 675 μl Tris-HCl buffer, 125 μl NBT, 125 μl NADH and 125 μl PMS were prepared. Moreover, ellagic acid (Merck KGaA) was used as a positive control. The samples were vortexed vigorously and incubated in the dark at RT for 5 min. The OD was then measured at 560 nm using a UV/Visible spectrophotometer (U-1500, Hitachi. Ltd.). The % RSC was calculated using the aforementioned equation. The IC50 value was calculated from the linear regression curve by plotting the % RSC against the corresponding concentrations. All analyses were carried out in triplicate and at least in two separate occasions.

Reducing power assay. The reducing power was evaluated on the basis of the method described in the study by Yen and Duh (29). In detail, 50 μl of each wine sample (0.25-8 μl/ml for red wines and 1.25-40 μl/ml for white wines) diluted in phosphate buffer (0.2 M, pH 6.6) was added to test tubes and mixed with 200 μl phosphate buffer and 250 μl of 1% w/v potassium ferricyanide $\{K_3[Fe(CN)_6]\}$ (PanReac AppliChem, ITW Reagents) in dH_2O. In each experiment, a blank containing 500 μl phosphate buffer and a negative control containing 250 μl phosphate buffer and 250 μl of 1% w/v potassium ferricyanide in dH_2O were prepared. Additionally, vitamin C was used as a positive control. The samples were vortexed vigorously and incubated at 50˚C for 20 min. Subsequently, 250 μl of 10% trichloroacetic acid (TCA; Merck KGaA) were added to the mixture and the samples were centrifuged (875 x g, 10 min, 25˚C). Following centrifugation, 700 μl of the supernatant was transferred to new test tubes and 250 μl dH_2O and 50 μl of 0.1% iron (III) chloride (Merck KGaA) in dH_2O were added. The samples were vortexed and incubated in the dark at RT for 10 min. Finally, the OD was measured at 700 nm using a UV/Visible spectrophotometer (U-1500, Hitachi, Ltd.). An absorbance unit 0.5 (AU0.5) value was calculated from the linear regression curve by plotting the OD at 700 nm against the corresponding concentrations. The AU0.5 value represents the concentration of the wine sample required to achieve an OD of 0.5 at 700 nm. All analyses were carried out in triplicate and at least in two separate occasions.

Cupric ion reducing antioxidant capacity (CUPRAC) assay. The reducing ability against copper ions was evaluated on the basis of the method described in the study by Apak *et al* (30). More specifically, 250 μl copper (II) chloride dihydrate ($CuCl_2$) solution (0.01 M; (Merck KGaA), 250 μl neocuproine (Nc) ethanolic solution (0.0075 M; Merck KGaA), 250 μl ammonium acetate ($NH_4CH_3CO_2$) solution (1 M, pH 7.0; Honeywell Research Chemicals), 225 μl dH_2O, and 50 μl of each wine sample (0.25-8 μl/ml for red wines and 1.25-40 μl/ml for white wines) diluted in dH_2O were mixed in test tubes. In each experiment, a blank comprising 250 μl $CuCl_2$ solution, 250 μl $NH_4CH_3CO_2$ solution and 525 μl dH_2O, and a negative control containing 250 μl $CuCl_2$ solution, 250 μl Nc solution, 250 μl $NH_4CH_3CO_2$ solution and 275 μl dH_2O were prepared. Furthermore, vitamin C was used as a positive control. The mixture was vortexed vigorously and incubated in the dark at RT for 30 min. The optical density was then measured at 450 nm using a UV/Visible spectrophotometer (U-1500, Hitachi, Ltd.). An AU0.5 value was calculated from the linear regression curve by plotting the OD at 450 nm against the corresponding concentrations. All analyses were carried out in triplicate and at least in two separate occasions.

Plasmid DNA relaxation assay. The protective ability against oxidative DNA damage was evaluated on the basis of the method previously described by Paul *et al* (31). More specifically, 3 μl of each wine sample (0.5-8 μl/ml for red wines and 2.5-40 μl/ml for white wines) diluted in sterilized dH_2O was mixed with 2 μl plasmid DNA pBluescript II SK (+) (3.2 μg) (Stratagene, Agilent Technologies), 1 μl of sterilized phosphate-buffered saline (PBS; 0.01 M, pH 7.4) (Gibco, Thermo Fischer Scientific, Inc.) and 4 μl of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; 95 mM; Merck KGaA) in sterilized PBS. A negative control comprising 8 μl sterilized PBS and 2 μl plasmid DNA, and a positive control comprising 4 μl sterilized PBS, 2 μl plasmid DNA and 4 μl of AAPH were also prepared. Vitamin C was used as a standard. Furthermore, the plasmid DNA was treated with the highest concentration of each wine sample in order to assess its effects on supercoiled conformation. The samples were vortexed and incubated for 45 min at 37˚C for the thermal decomposition of AAPH and the generation of peroxyl radicals ($ROO^{\bullet}$). Following incubation, 3 μl of loading buffer were added and the samples were loaded on a 0.8% agarose gel (SERVA Electrophoresis GmbH), stained with ethidium bromide (10 μg/ml) for 30 min at RT and electrophoresed at 70 V for 60 min. The gel was then exposed to UV using the MultiImage Light Cabinet (ProteinSimple). The image was captured and analyzed using a quantification software (AlphaView software, AlphaInnotech). The protective ability of the wine samples was calculated through the following equation:

$$\%\,Inhibition = \frac{(S - So)}{(Scontrol - So)} \times 100$$

where 'S' stands for the percentage of supercoiled plasmid DNA in samples, 'So' stands for the percentage of supercoiled plasmid DNA in the positive control, and 'Scontrol' stands for the percentage of supercoiled plasmid DNA in the negative control.

The IC50 value was calculated from the linear regression curve by plotting the % inhibition against the corresponding concentrations. All analyses were conducted at least in two separate occasions.

Statistical analysis. One-way analysis of variance (ANOVA), followed by the Holm-Sidak post hoc test, were performed for multiple pairwise comparisons between the mean IC50 or AU0.5 values of the different wine varieties. An unpaired t-test was performed to compare the mean IC50 or AU0.5 values between the red and white wine varieties. Pearson's correlation coefficient (r) was calculated to determine the correlation between the TPC and antioxidant capacity of the red and white wine varieties. All data are presented as the mean ± standard error of the mean (SEM). A value of $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, Inc.

Results

TPC and antioxidant capacity of the different wine varieties. According to the results obtained, all wine varieties exhibited potent antioxidant activities (Fig. 1). Among them, the red wine variety Xinomavro demonstrated the most potent antioxidant capacity, an assertion supported by the lowest IC50 or AU0.5 values in all cell-free assays tested. By contrast, the white wine variety Malagouzia exhibited the weakest antioxidant properties, as denoted by the highest IC50 or AU0.5 values, in all cell-free assays examined.

To begin with TPC, measured using the Folin-Ciocalteu method and presented in Fig. 1A, Xinomavro exhibited the highest TPC, which was calculated at 1.602±0.106 mg GAE/ml, whereas Malagouzia exhibited the lowest, which was calculated at 0.178±0.013 mg GAE/ml. The TPC of Agiorgitiko was calculated at 1.291±0.072 mg GAE/ml and that of Assyrtiko was calculated at 0.284±0.072 mg GAE/ml. The statistical analysis revealed significant differences between the red and white wine varieties, as well as a significant difference between Xinomavro and Agiorgitiko wine varieties.

As regards $ABTS^{\bullet+}$ radical scavenging assay, illustrated in Fig. 1B, Xinomavro exhibited the highest capacity to neutralize the corresponding free radicals, whereas Malagouzia demonstrated the lowest. More specifically, the IC50 value calculated for Xinomavro was 0.985±0.153 μl/ml, that for Agiorgitiko was 6.441±2.114 μl/ml, that for Assyrtiko was 5.004±1.385 μl/ml and that for Malagouzia was 14.29±3.145 μl/ml. A finding of particular interest was that the IC50 value of Assyrtiko was lower than that of Agiorgitiko. The statistical analysis revealed a significant difference between all wine varieties and Malagouzia. Moreover, the IC50 value calculated for vitamin C was 2.298±0.044 μg/ml (data not shown).

As regards the $DPPH^{\bullet}$ radical scavenging assay depicted in Fig. 1C, Xinomavro exhibited the highest efficacy to scavenge the corresponding free radicals, whereas Malagouzia exhibited the lowest. In particular, the IC50 value calculated for Xinomavro was 1.591±0.260 μl/ml, that for Agiorgitiko was 2.210±0.176 μl/ml, that for Assyrtiko was 12.870±1.808 μl/ml and that for Malagouzia was 22.200±3.046 μl/ml. The statistical analysis revealed significant differences between tje red and white wine varieties, as well as a significant difference between the Assyrtiko and Malagouzia wine varieties. In addition, the IC50 value calculated for vitamin C was 4.565±0.183 μg/ml (data not shown).

As regards the $O_2^{\bullet-}$ radical scavenging assay, illustrated in Fig. 1D, Xinomavro demonstrated the most potent ability to scavenge corresponding free radicals, while by contrast, Malagouzia exhibited the weakest. In particular, the IC50 value for Xinomavro was calculated as 2.277±0.260 μl/ml, that for Agiorgitiko was calculated as 3.076±0.300 μl/ml, that for Assyrtiko was calculated as 8.550±0.503 μl/ml and that for Malagouzia was calculated as 12.34±2.08 μl/ml. The statistical analysis revealed significant differences between the red and white wine varieties, as well as a significant difference between the Assyrtiko and Malagouzia wine varieties. Additionally, the IC50 value calculated for ellagic acid was 255.430±8.500 μg/ml (data not shown).

In terms of the reducing power assay, presented in Fig. 1E, Xinomavro exhibited the most prominent reducing properties, whereas Malagouzia exhibited the weakest. In particular, the AU0.5 value calculated for Xinomavro was 1.091±0.100 μl/ml, that for Agiorgitiko was 1.548±0.077 μl/ml, that for Assyrtiko was 8.361±0.909 μl/ml and that for Malagouzia was 12.08±0.974 μl/ml. Statistically significant differences were observed between the red and white wine varieties. Additionally, a statistically significant difference was observed between the Assyrtiko and Malagouzia wine varieties. Moreover, the AU0.5 value calculated for vitamin C was 1.700±0.062 μg/ml (data not shown).

As regards the CUPRAC assay, presented in Fig. 1F, Xinomavro exhibited the highest reducing properties, whereas Malagouzia exhibited the lowest. To be more specific, the AU0.5 value calculated for Xinomavro was 1.531±0.140 μl/ml, that for Agiorgitiko was 2.105±0.188 μl/ml, that for Assyrtiko was 8.552±0.798 μl/ml and that for Malagouzia was 16.24±1.292 μl/ml. The statistical analysis revealed significant differences between the red and white wine varieties, as well as a significant difference between the Assyrtiko and Malagouzia wine varieties. Moreover, the AU0.5 value calculated for vitamin C was 6.551±0.050 μg/ml (data not shown).

Finally, concerning the plasmid DNA relaxation assay, depicted in Fig. 1G, Xinomavro exhibited the highest efficacy to inhibit the formation of the corresponding free radicals, whereas Malagouzia demonstrated the lowest. More elaborately, the IC50 value calculated for Xinomavro was 2.078±0.163 μl/ml, that for Agiorgitiko was 2.547±0.218 μl/ml, that for Assyrtiko was 15.70±1.125 μl/ml and that for Malagouzia was 19.62±1.830 μl/ml. The statistical analysis revealed significant differences between the red and white wine varieties, as well as a significant difference between the Assyrtiko and Malagouzia wine varieties. In addition, the IC50 value calculated for vitamin C was 300.302±21.852 μg/ml (data not shown).

TPC and antioxidant capacity of the red and white wine varieties. In order to compare the TPC and the antioxidant properties between the red and white wine varieties, the IC50 or AU0.5 values of the wine samples of the red or white wine varieties were pooled together and the mean IC50 or AU0.5 values were calculated. According to the results obtained, substantial differences were observed between the red and white wine varieties. To be more specific, the red wine varieties demonstrated a higher phenolic content and more potent antioxidant activities than the white wine varieties, and all differences were statistically significant (Fig. 2).

As regards the Folin-Ciocalteu assay, illustrated in Fig. 2A, the TPC for the red wine varieties was calculated 1.446±0.074 mg GAE/ml and that for the white wine varieties was estimated at 0.231±0.019 mg GAE/ml. As regards the $ABTS^{\bullet+}$ radical scavenging assay, presented in Fig. 2B, the IC50 value for the red wine varieties was estimated at 3.713±1.243 μl/ml and that for the white wine varieties was estimated at 9.648±2.048 μl/ml. Concerning the $DPPH^{\bullet}$ radical scavenging assay (Fig. 2C), the IC50 value for the red wine varieties was calculated at 1.900±0.171 μl/ml and that for the white wine varieties was calculated at 17.530±2.093 μl/ml. In terms of the $O_2^{\bullet-}$ radical scavenging assay, presented in Fig. 2D, the IC50 value for the red wine varieties was calculated at 2.676±0.218 μl/ml, while the IC50 value for the white wine varieties was calculated at 10.45±1.144 μl/ml.

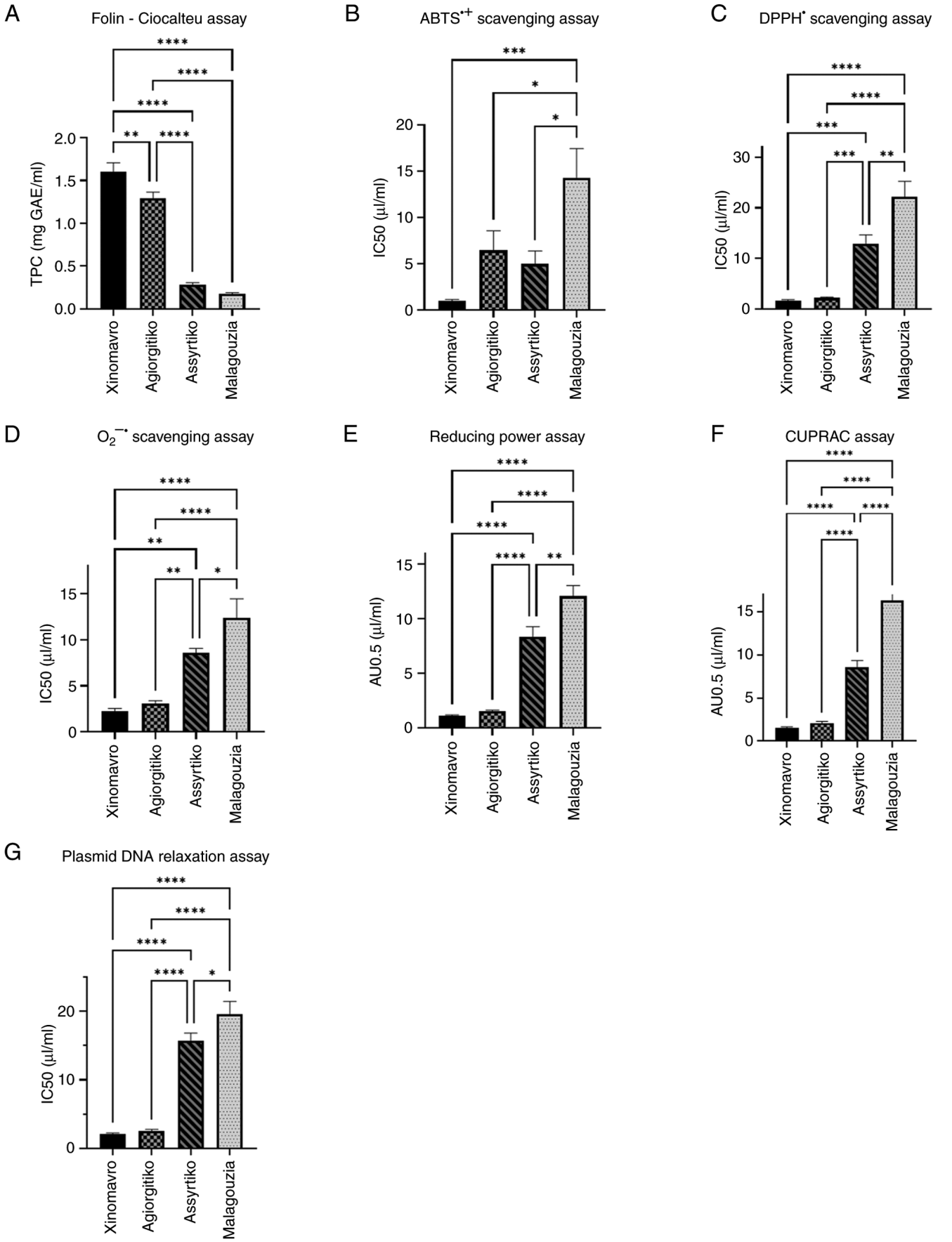

Figure 1. TPC and the antioxidant activities of Xinomavro, Agiorgitiko, Assyrtiko and Malagouzia wine varieties determined using (A) Folin-Ciocalteu assay, (B) $ABTS^{\bullet+}$ scavenging assay, (C) $DPPH^{\bullet}$ scavenging assay, (D) $O_2^{\bullet-}$ scavenging assay, (E) reducing power assay, (F) CUPRAC assay, and (G) plasmid DNA relaxation assay. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$. TPC, total phenolic content; $ABTS^{\bullet+}$, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; $O_2^{\bullet-}$, superoxide radical; CUPRAC, cupric ion reducing antioxidant capacity IC50, half-maximal inhibitory concentration; AU0.5, absorbance unit 0.5.

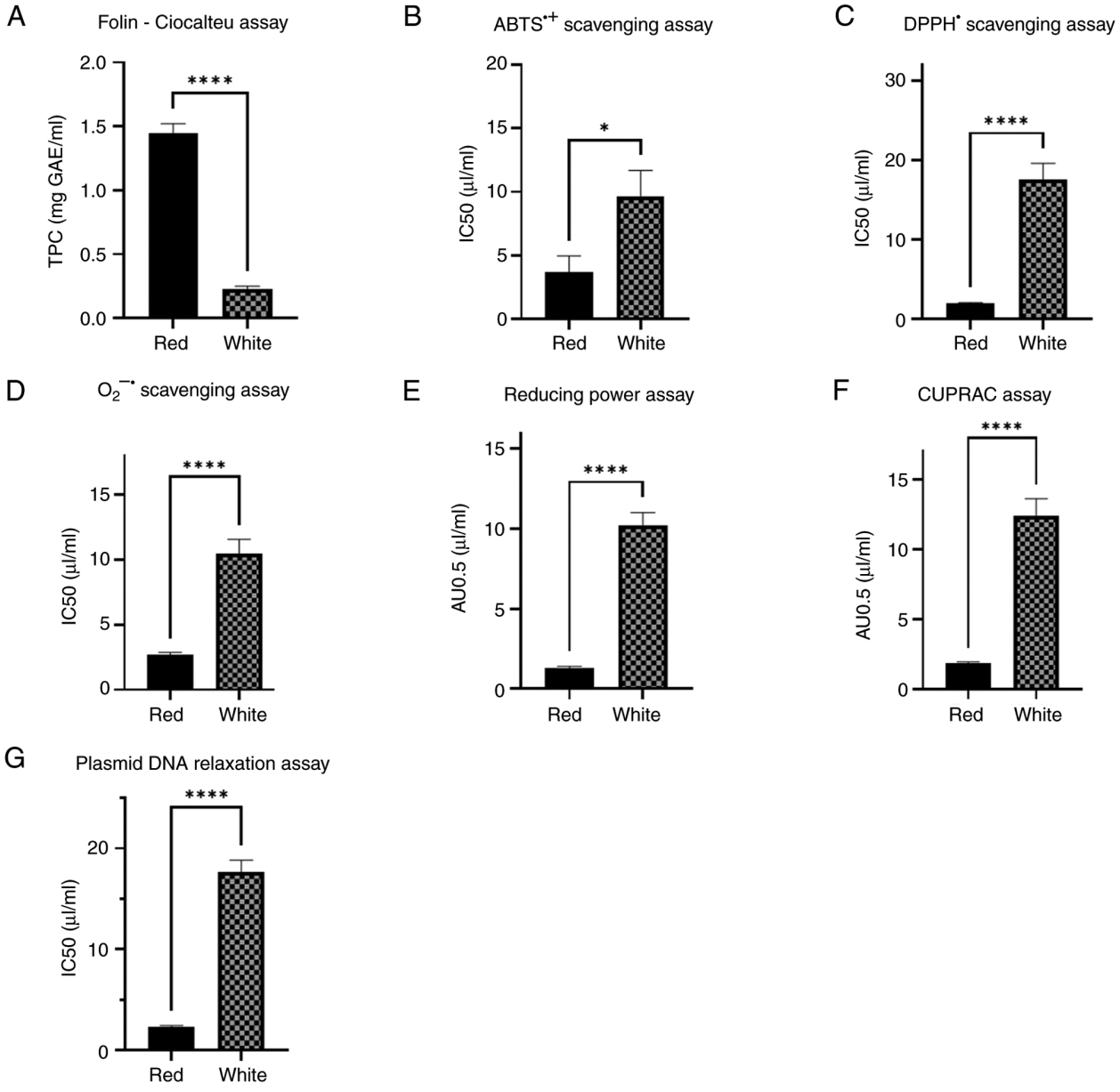

Figure 2. TPC and the antioxidant activities of the red and white wine varieties determined using the (A) Folin-Ciocalteu assay, (B) ABTS$^{\bullet+}$ scavenging assay, (C) DPPH$^{\bullet}$ scavenging assay, (D) $O_2^{\bullet-}$ scavenging assay, (E) reducing power assay, (F) CUPRAC assay, and (G) plasmid DNA relaxation assay. *P<0.05 and ****P<0.0001. TPC, total phenolic content; ABTS$^{\bullet+}$, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical; DPPH$^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; $O_2^{\bullet-}$, superoxide radical; CUPRAC, cupric ion reducing antioxidant capacity IC50, half-maximal inhibitory concentration; AU0.5, absorbance unit 0.5.

With respect to the reducing power assay, illustrated in Fig. 2E, the AU0.5 value for the red wine varieties was calculated at 1.319±0.085 μl/ml and that for the white wine varieties was calculated at 10.22±0.803 μl/ml. Concerning the CUPRAC assay (Fig. 2F), the AU0.5 value for the red wine varieties was estimated at 1.818±0.135 μl/ml and that for the white wine varieties was estimated at 12.39±1.234 μl/ml.

Finally, as regards the antigenotoxic properties, evaluated using plasmid DNA relaxation assay and presented in Fig. 2G, the IC50 value for the red wine varieties was calculated at 2.312±0.145 μl/ml and that for the white wine varieties was calculated at 17.66±1.155 μl/ml.

Correlation between the TPC and antioxidant capacity of the red and white wine varieties. Pearson's correlation coefficient (r) was calculated to determine the correlation between the TPC and antioxidant properties of the red and white wine varieties. The correlation analysis demonstrated significant negative correlations in almost all cases, indicating that the higher the TPC, the lower the IC50 or AU0.5 value, and as a result, the more potent the antioxidant capacity.

As regards the red wine varieties (Fig. 3), significant negative correlations were observed between the TPC and ABTS$^{\bullet+}$ radical scavenging assay (r=-0.5580l; Fig. 3A), the $O_2^{\bullet-}$ radical scavenging assay (r=-0.5881; Fig. 3C), the reducing power assay (r=-0.5009; Fig. 3D), the CUPRAC assay (r=-0.5965; Fig. 3E) and the plasmid DNA relaxation assay (r=-0.5381; Fig. 3F). The negative correlation detected between TPC and DPPH$^{\bullet}$ radical scavenging assay (r=-0.3093; Fig. 3B) was not significant.

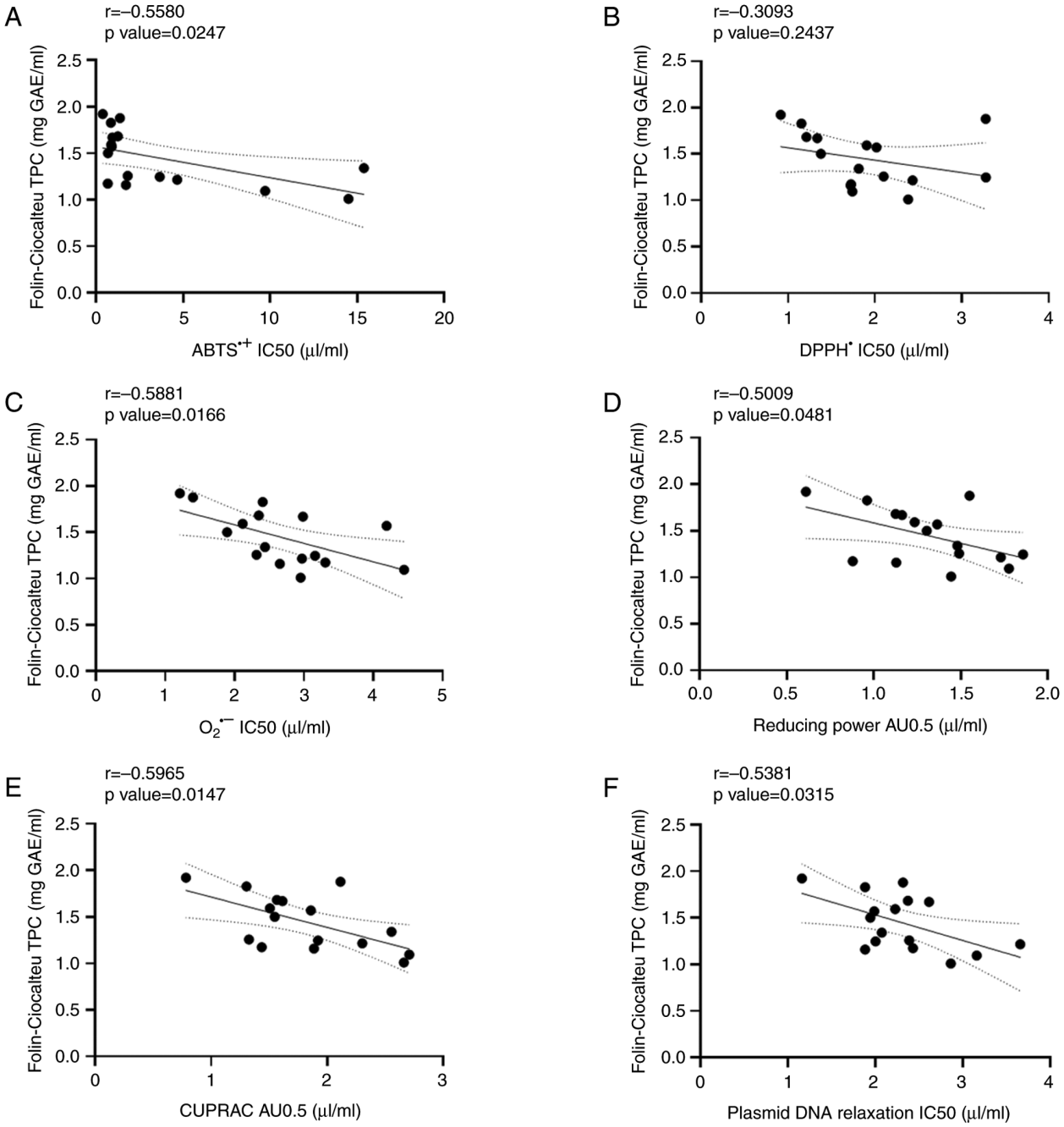

Figure 3. Correlation analysis between TPC and (A) ABTS$^{\bullet+}$ scavenging assay, (B) DPPH$^{\bullet}$ scavenging assay, (C) $O_2^{\bullet-}$ scavenging assay, (D) reducing power assay, (E) CUPRAC assay, and (F) plasmid DNA relaxation assay in the red wine varieties. TPC, total phenolic content; ABTS$^{\bullet+}$, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical; DPPH$^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; $O_2^{\bullet-}$, superoxide radical; CUPRAC, cupric ion reducing antioxidant capacity IC50, half-maximal inhibitory concentration; AU0.5, absorbance unit 0.5.

Concerning the white wine varieties (Fig. 4), significant negative correlations were observed between TPC and ABTS$^{\bullet+}$ radical scavenging assay (r=-0.6584; Fig. 4A), the DPPH$^{\bullet}$ radical scavenging assay (r=-0.6119; Fig. 4B), the $O_2^{\bullet-}$ radical scavenging assay (r=-0.6740; Fig. 4C), the reducing power assay (r=-0.8329; Fig. 4D), the CUPRAC assay (r=-0.8539; the Fig. 4E) and the plasmid DNA relaxation assay (r=-0.5693; Fig. 4F).

Discussion

In the present study, the phenolic content and the antioxidant potency of four native Greek wine varieties, and more specifically, the red wine varieties, Xinomavro and Agiorgitiko, and the white wine varieties, Assyrtiko and Malagouzia, were thoroughly evaluated using a methodology previously proposed by the authors (24). The utilization of a panel of reliable and valid *in vitro* cell-free assays represents the first line of screening towards the investigation of the antioxidant activities of natural products that are particularly rich in polyphenolic compounds. It has been well-established that antioxidants exert their protective effects by acting as free radical scavengers, as reducing agents, as metal chelators and as enhancers of antioxidant gene expression (32,33). For this reason, in the present study, the antioxidant activities of Greek

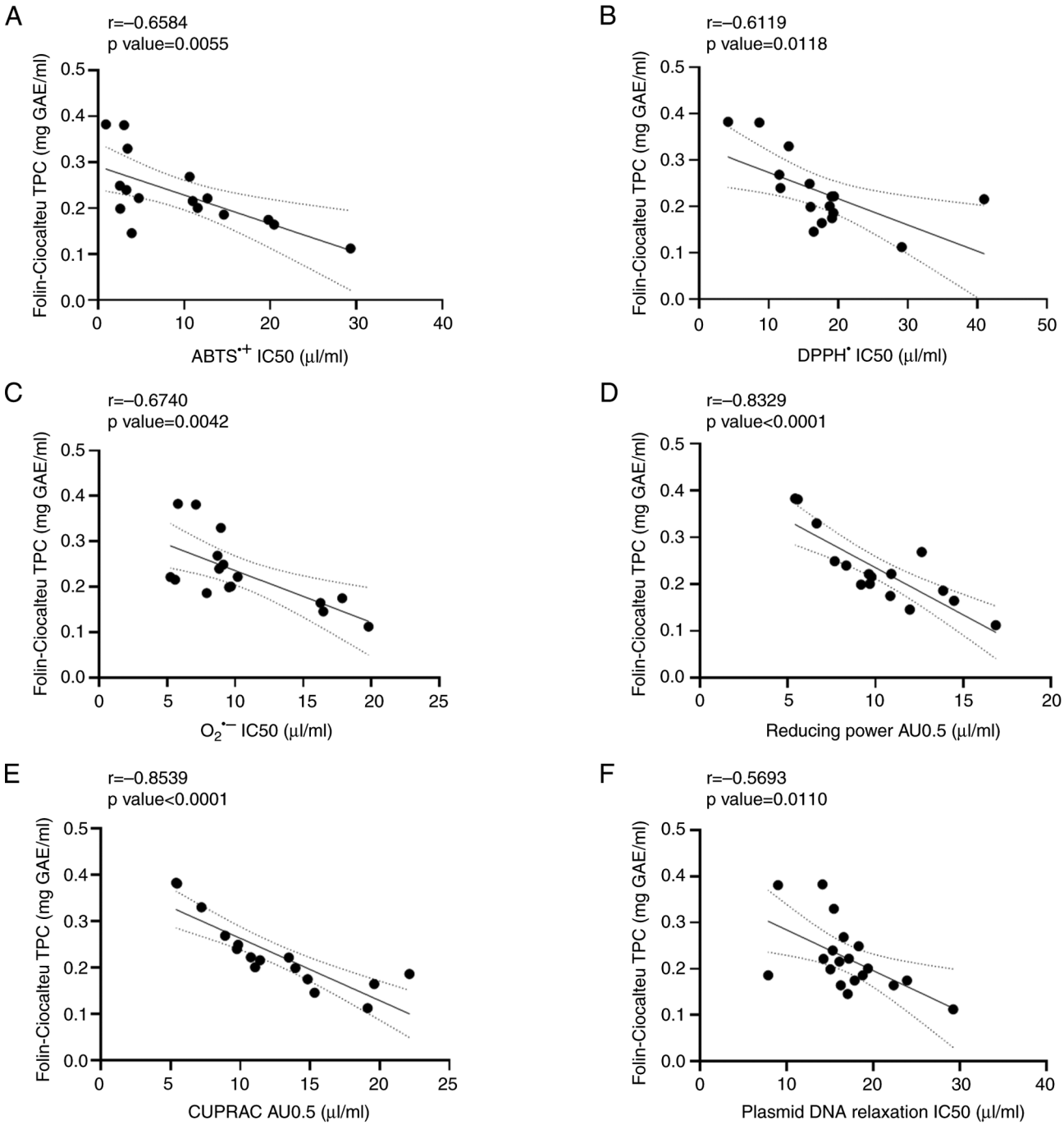

Figure 4. Correlation analysis between TPC and (A) $ABTS^{\bullet+}$ scavenging assay, (B) $DPPH^{\bullet}$ scavenging assay, (C) $O_2^{\bullet-}$ scavenging assay, (D) reducing power assay, (E) CUPRAC assay, and (F) plasmid DNA relaxation assay in the white wine varieties. TPC, total phenolic content; $ABTS^{\bullet+}$, 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) radical; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; $O_2^{\bullet-}$, superoxide radical; CUPRAC, cupric ion reducing antioxidant capacity IC50, half-maximal inhibitory concentration; AU0.5, absorbance unit 0.5.

wine varieties were assessed multifacetedly on the basis of their antiradical, reducing, and antigenotoxic properties.

Polyphenolic compounds are the main bioactive phytochemicals of wine that exhibit antioxidant potency (34). More specifically, polyphenols protect wine from oxidation, thus extending its shelf life, and exert health-promoting effects in biological systems (35-38). However, the polyphenolic content and profile differs significantly between the red and white wine varieties and, in general, red wine varieties exhibit higher concentrations of phenolic compounds than the white wine varieties (12,39). In particular, the concentration of phenolic compounds in red wines is ~6-fold higher than that in white wines, due to the fact that the red juice is fermented with the grape skins and seeds, wherein the phenolic compounds are mainly concentrated (40). As regards the polyphenolic profile, tannins and anthocyanins are the most abundant polyphenols in red wines, whereas white wines are particularly rich in phenolic acids (41).

The first objective of the present study was to determine the polyphenolic content of the four native Greek wine varieties using the Folin-Ciocalteu method. According to the results obtained, the TPC of red wine varieties was significantly

higher than that of the white wine varieties, a finding supported by scientific literature (42-47). As mentioned above, the main reason for the higher levels of polyphenolic compounds in red wines is that the fermentation process of red juice includes all grape parts, resulting in greater polyphenol extraction in the final product (48). Furthermore, in the present study, as regards the TPC of different wine varieties, Xinomavro demonstrated the highest concentration of polyphenolic compounds, whereas Malagouzia exhibited the lowest. A significant difference was also detected between the TPC of the Xinomavro and Agiorgitiko red wine varieties, with Xinomavro exhibiting higher levels of polyphenols. A previous study by the authors investigated the phenolic content and the antioxidant and antimutagenic properties of wine extracts derived from Xinomavro, Agiorgitiko, Assyrtiko and Malagouzia wine varieties, using a battery of *in vitro* cell-free assays (49). That study reported that the TPC of the red wine extracts was higher than that of the white wine extracts; however, there were no significant differences between the TPC of Xinomavro and Agiorgitiko wine extracts (49).

The quantitative and the qualitative polyphenolic profile determines the biological actions of wine (34,50). For the purpose of evaluating the bioactive load of polyphenolic compounds, the antioxidant activities of the four native Greek wine varieties were assessed on the basis of their free radical scavenging capacity and their reducing properties. According to the results obtained, the red wine varieties demonstrated more potent antioxidant activities than the white wine varieties in all cell-free assays examined. This finding may be attributed to the higher polyphenolic content of the red wine varieties, which is in accordance with findings obtained from previous studies (51-55). Moreover, in the present study, concerning the antioxidant activities of the different wine varieties, Xinomavro exhibited the strongest antioxidant efficacy, whereas Malagouzia demonstrated the weakest in all cell-free assays tested. The obtained results are in line with the findings obtained using the Folin-Ciocalteu method, with Xinomavro wine variety exhibiting the highest TPC and Malagouzia wine variety the slightest.

Finally, the present study investigated the correlation between the TPC and antioxidant activities of the red and white wine varieties. The Pearson's correlation coefficient (r) revealed significant negative correlations between the TPC and antioxidant assays in almost all cases, suggesting that the antioxidant properties of red and white wine varieties are significantly affected by polyphenolic content. In particular, the higher the TPC, the greater the antioxidant activities. As regards the red wine varieties, similar levels of significant strong negative correlations were observed between the TPC and all antioxidant assays, apart from the DPPH$^{\bullet}$ scavenging assay, wherein the negative correlation was not significant. Concerning the white wine varieties, significant strong negative correlations were observed between the TPC and all antioxidant assays. A finding of particular interest was that the highest degree of correlation was observed between the TPC, and the reducing power and CUPRAC assays. To be more specific, these *in vitro* test tube assays examine the ability of a sample to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions and cupric (Cu^{2+}) to cuprous (Cu^{1+}) ions, respectively. Reducing agents are strong electron donors, that can reduce oxidized intermediates of lipid peroxidation process in biological systems (56).

Prior to summarizing the conclusions of the particular research, it should be underlined that the findings need to be viewed in light of some limitations. As previously stated, the present study represents the first-line test for evaluating the phenolic content and antioxidant capacity of commercial wines of Agiorgitiko, Xinomavro, Assyrtiko and Malagouzia. Hence, it provides some early, yet valuable, indications of the bioactivity of these indigenous Greek varieties. It has to be mentioned that at this phase of the research project, we did not perform liquid chromatography-high-resolution mass spectrometry to identify and characterize the main phenolics of the selected wines in order to correlate them with their antioxidant properties. Furthermore, the sugar content and flavor are both variables that could have been considered in the determination of the antioxidant potency of wines. To the best of our knowledge, high performance liquid chromatography (HPLC), Fehling's method for reducing sugars and various enzymatic analyses are widely used for the determination of the sugar content; however, no such investigations have been conducted in the present study. Finally, the volatile composition of wine is closely related to its characteristic aroma and flavor (57). Among the other volatile flavor compounds, esters, formed by reactions occurring between alcohols and acids, are responsible for the primary fruit and floral aromas and flavors in wines (58,59). However, the present study did not perform any analysis to determine the volatile composition of the selected wines and, therefore, it is not safe to draw any conclusions as regards the effect of wine flavor on the parameters measured.

Conclusively, the present study reported that the indigenous Greek wine varieties, Xinomavro, Agiorgitiko, Assyrtiko and Malagouzia, are rich sources of phytochemical constituents and exert strong biological activities, attributed to their polyphenolic content. The red wine varieties demonstrate a higher TPC and exhibit more prominent antioxidant activities than the white wine varieties. In addition, among the different wine varieties, Xinomavro exhibited the highest concentration of phenolic compounds and the most robust antioxidant activities. Overall, the findings of the present study dictate that these native Greek wines are highly bioactive, particularly Xinomavro, and their moderate consumption may be related to beneficial health effects, due to their potent antioxidant properties.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FT and DK conceptualized the study. FT and MG were involved in the study methodology. FT, PV and ZS were

involved in the formal analysis and in data curation. FT, MG and PV were involved in the writing and preparation of the original draft. PV was involved in the writing, reviewing and editing of the manuscript. FT was involved in visualization (creation of the figures for the article). DK supervised the study and was involved in project administration. FT and DK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

DK is an Editor of the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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