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Decrease of Heat Shock Protein Levels and Cell Populations by Wine Phenolic Extracts

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The effect of red and white wine total extracts and phenolic fractions on heat shock protein (Hsp) levels in tumor cells and on tumor and endothelial cell populations in vitro has been investigated. Total extracts of red wines decreased Hsp70 and Hsp27 levels and the numbers of tumor and endothelial cells. Several red and white wine fractions significantly decreased Hsp27 levels, and some of them had also an effect on Hsp70 levels. A red wine fraction rich in polymeric flavanols and a white wine one rich in phenolic acids, flavonols, and tyrosol strongly lowered Hsp27 levels. Some red and white wine fractions strongly reduced tumor cell numbers, whereas most of them decreased endothelial cell numbers to variable extents. The present results indicate that wine phenolics decrease Hsp levels in tumor cells and tumor and endothelial cell populations. These properties may be important in the potent anticarcinogenic action of wine phenolics.

KEYWORDS: Heat shock proteins; Hsp70; Hsp27; tumor cells; endothelial cells; polyphenols; phenolic compounds; wine

INTRODUCTION

Wine, notably red wine, is rich in phenolic compounds, which are implicated in the inhibition of tumor and endothelial cell proliferation (*1*−*4*). Moreover, some plant-derived phenolics decrease heat shock protein synthesis (*5*, *6*).

Heat shock proteins (Hsps) are a group of proteins induced by heat shock or other forms of stress in all cells or organisms. Hsps include a number of different molecular weight families: Hsp110, Hsp90, Hsp70, Hsp60, Hsp40, and Hsp27 (*7*). Hsps protect cells from irreversible damage during cellular injury by refolding damaged proteins and thus assisting in the recovery from stress (*8*). Under normal growth conditions, an Hsp functions in the correct folding of newly synthesized polypeptides and the translocation of intracellular proteins across membranes (*8*, *9*). Hsp synthesis is activated as a common and ubiquitous response to a wide range of conditions and agents in addition to thermal stress (*10*). Hsp overexpression is also associated with many pathological states and human diseases such as ischemia/reperfusion, fever, inflammation, and cancer (*11*). In particular, Hsp70 and Hsp27 are frequently overexpressed in human cancers and may contribute to carcinogenesis (*11*, *12*). Furthermore, Hsp70 and Hsp27 play an inhibitory role in apoptosis (*13*, *14*). Although a number of chemicals are known to induce the synthesis of Hsps, only very few agents are known to inhibit their synthesis (*10*, *15*). Some phenolics of plant origin, such as quercetin, flavone, genistein, and kaempferol, decrease the levels of Hsps (*5*, *6*).

Dietary factors contribute to about a third of potentially preventable cancers (*16*), and the long-known preventive effect of plant-based diets on tumorigenesis is well documented (*17*). At least part of the protective effect of dietary factors on cancers is attributed to dietary phenolics. However, the mechanism of action of the protective effects is often heterogeneous and is exerted simultaneously at several levels. Indeed, phenolic compounds inhibit a multitude of kinases intercepting the signaling cascades of several growth factors. For instance, flavonoids, an important class of dietary phenolics, are competitive inhibitors with respect to the well-conserved ATP binding site of kinases (*18*) intercepting the signaling cascades of several growth factors. Thus, flavonoids are able to inhibit both tumor cell proliferation and angiogenesis, processes regulated by different growth factors in cells of different differentiation backgrounds, that is, tumor versus endothelial cells (*19*). Red wine polyphenols have been reported to inhibit tumor cell proliferation (*2*, *4*), and the red wine constituent resveratrol suppresses endothelial cell proliferation (*1*). Moreover, resveratrol reduces carcinogenesis in animal models (*20*, *21*).

Wine phenolics originate mainly from grapes and also from yeast and wood. Most phenolics, however, are gradually converted during winemaking to other phenolic species. Wine phenolics are divided into flavonoids and non-flavonoids. The family of flavonoids includes mainly flavonols, flavanols, and anthocyanins, whereas the non-flavonoids mainly include phe-

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nolic acids (benzoic and hydroxycinnamic acids) and stilbenes. Red wines contain all of the above phenolics, whereas white wines contain mainly phenolic acids and flavonols (*22*, *23*). Red winemaking includes the procedure of maceration, a process that is not included in white winemaking as skins are removed during the vinification of white wine. Thus, white wines obtained by free-run press contain essentially hydroxycinnamic acids, which are the only phenolics present in the pulp. On the other hand, wine prepared with pomace contact, and in particular red wines, also show large amounts of flavonoids, extracted from the solid parts of the cluster (*24*, *25*).

As phenolic compounds have been reported to act as inhibitors of Hsp synthesis, and as antiproliferative agents, it appeared to be important to investigate the effects of various wine extracts on the levels of Hsp70 and Hsp27 in a tumor cell line and on the populations of one tumor and one endothelial cell line.

MATERIALS AND METHODS

Wines and Wine Sample Preparation. The 12 wines examined were commercial wines of the Greek vineyard and were made from the following grape varieties: 1, Mavrodaphne, sweet red wine (Mavrodaphne Patras, Achaia Clauss, 1997); 2, Xinomavro, dry red wine (Naoussa Boutaris, 1997); 3, Agiorgitiko, dry red wine (Agiorgitiko Boutaris, 1997); 4, Limnio, dry red wine (Limnio Karras, 1997); 5, Syrah, dry red wine (Syrah Ktima Kyr-Yianni, 1997); 6, Debina, dry white wine (White dry Zitsa, Union of Agricultural Cooperatives of Ioannina, 1998); 7, Roditis, dry white wine (Orinos Roditis, Grypas, 1999); 8, Athiri, dry white wine (Athiri Vounoplagias, Rhodes, 1999); 9, Muscat, dry white wine (Choise of Kalaitzis, Union of Agricultural Cooperatives of Lemnos, 1999); 10, Robola, dry white wine (Robola Kephallonias, 1998); 11, Sauvignon Blanc, dry white wine (Sauvignon Blanc, Grypas, 1999); 12, Traminer, dry white wine (Traminer, Katogi Averof, 1999).

The 12 wines were analyzed for total phenolics, whereas total wine extracts were used for biological tests. Total wine extracts were prepared by rotary evaporation at 25 °C at 80 mbar. The dealcoholated and dehydrated residue was redissolved in DMSO.

Xinomavro and Roditis experimental wines were also used. In Xinomavro winemaking the maceration procedure was applied, whereas in Roditis winemaking the free-run juice was used. In both cases, young wines, that is, wines before aging in barrels, were used to evaluate the biological properties of wine phenolics of different grape origins.

Xinomavro red wine is a well-known Greek appelation of origin wine, whereas Roditis white wine is a well-known Greek table wine.

Total wine extracts (Xinomavro, X; and Roditis, R) were dealcoholated wines concentrated by rotary evaporation at 25 °C at 80 mbar. Dealcoholated wines were obtained by evaporation; wine added to an equal volume of distilled water was concentrated to the original volume (25 °C, 80 mbar) to remove the alcohol without destroying the phenolic compounds (*26*).

Liquid−liquid extractions were performed to obtain three extracts containing different classes of polyphenols (*27*). The dealcoholized wine (pH 2.0) was first extracted with ethyl acetate. The aqueous phase was the first fraction (X1 or R1). The organic phase after evaporation was redissolved in water at pH 7.0 and extracted again with ethyl acetate. This organic phase was the second fraction (X2 or R2). The aqueous phase was adjusted at pH 2.0 and extracted again with ethyl acetate. This extract was the third fraction (X3 or R3). Each of the three fractions was subfractionated into nonpolymeric (monomeric and dimeric) and polymeric polyphenols using a Sephadex LH-20 column (*28*). Nonpolymeric polyphenols (subfraction a, X1a/X2a/X3a or R1a/R2a/R3a) were desorbed by methanol/acetic acid from the gel and polymeric ones (subfraction b, X1b/X2b/X3b or R1b/R2b/R3b) by acetone/acetic acid. It should be noted that subfractions X2b and X3b or R2b and R3b are likely of limited molecular weight as they were extracted in ethyl acetate and less polymeric than X1b or R1b, respectively.

In phenolic analyses samples in 10% ethanol were used, whereas in biological assays samples in DMSO were used.

Analysis of Phenolics. *Phenolic Content.* The total phenolic contents of the samples were determined according to the Folin−Ciocalteu method (*29*) using gallic acid as a standard.

Phenolic Composition. All samples were analyzed by high-performance liquid chromatography with a diode array detector (HPLC-DAD) for individual phenolic compounds.

Samples were filtered using a syringe filter (PTFE 0.45, Alltech) prior to the injection.

A Waters 600E system with a 996 photodiode array detector and a 600E pump was used. Chromatograms were treated using the Millenium 32 program. The column used was a C18 reversed phase Spherisorb (4.0 × 250 mm) with 5-μm packing (*30*).

The mobile phases were (A) water/glacial acetic acid (98:2), (B) methanol/water/glacial acetic acid (60:38:2), and (C) methanol/glacial acetic acid (98:2). The gradient used was as follows: 0−30 min, 100% A at 0.20 mL/min; 30−40 min, 58.3% A−41.7% B at 0.60 mL/min; 40−120 min, 41.7% A−58.3% B at 0.20 mL/min; 120−155 min, 25% A−75% B at 0.30 mL/min; 155−165 min, 100% C at 0.60 mL/min; 165−180 min, 100% C at 0.90 mL/min.

Chromatographic peaks were identified on the basis of the retention time and the UV−vis spectra of the standards used. In addition, the following absorbance characteristics of the phenolic classes, which were derived from the literature (*31*, *32*) and from our observations using the standards, were used: benzoic acids at 250−280 nm; hydroxycinnamic acids at 305−330 nm and several also at 290−300 nm; anthocyanins at 450−560 and 240−280 nm and some at 315−325 nm; flavanols at 270−280 and ∼230 nm; flavonols at 350−380 and 250−270 nm and some ∼300 nm; flavones and isoflavones at 300−350 and 245−270 nm; flavanones at 270−295 nm and some at 300−320 nm. Some peaks exhibited maximum of absorbance at 280−305 nm, and they were not classified into the above groups. They were expressed as unclassified 280−305 nm. Some of the standards used, such as aldehydes and *trans*-resveratrol, exhibited these characteristics. Some other peaks exhibited maximum absorbance at ∼230 nm, and they also absorbed at ∼280 nm. These peaks were expressed as unclassified 230 nm. Subsequently, all peaks were classified into nine groups. As main phenolic peaks were taken those exhibiting high area at 280, 255, 320, 360, or 520 nm.

The standard phenolic compounds used were as follows.

Benzoic acids: gallic acid (Sigma, St. Louis, MO), *p*-hydroxybenzoic acid (Sigma), protocatechuic acid (Sigma), vanillic acid (Sigma), syringic acid (Sigma), gentistic acid (Sigma), 2,3-dihydroxybenzoic acid (Sigma); and *p*-anisic acid (Sigma).

Cinnamic acids: caffeic acid (Sigma), chlorogenic acid (Sigma), ellagic acid (Sigma), *p*-coumaric acid (predominantly *trans*, Sigma), ferulic acid (Sigma), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (predominantly *trans*, Sigma), *o*-coumaric acid (predominantly *trans*, Sigma), 3,4-dihydroxyhydrocinnamic acid (Sigma), *trans*-cinnamic acid (Aldrich), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) (Sigma).

Anthocyanins: malvidin 3-glcucoside (Polyphenols), pelargonidine chloride (Sigma), cyanidin chloride (Extrasynthese).

Flavanols: (+)-catechin (Sigma), (−)-epicatechin (Sigma), (−)-epigallocatechin gallate (Sigma), procyanidin B_3 (Sigma).

Flavonols: rutin hydrate (Sigma), quercetin hydrate (Sigma), morin (Sigma), myricetin (Sigma), kaempferol (Fluka), galangin (Aldrich).

Flavones and isoflavones: apigenin (Sigma), luteolin (Extrasynthese), 5,7-dihydroxyflavone (Sigma), chrysin (Aldrich), 3-hydroxy-7-methoxyflavone (Sigma), 7-hydroxyflavone (Sigma), genistein (Sigma), 5,7-dihydroxy-4′-methoxyisoflavone (biochanin A) (Sigma).

Flavanones: hesperetin (Sigma), (±)-naringenin (Sigma), 2′-hydroxyflavanone (Sigma).

Aldehydes: 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde) (Aldrich), protocatechuic aldehyde, (Sigma), vanillin (Sigma), *trans*-3,5-dimethoxy-4-hydroxycinnamaldehyde (sinapaldehyde) (Aldrich).

Other phenolics: protocatechuic acid ethyl ester (Sigma), umbelliferone (Sigma), 4′-methoxychalcone (Aldrich), phloridzin (Sigma), tyrosol (Extasynthese), oleuropein (Extrasynthese), phloretin (Sigma), arbutin (Sigma), resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) (Sigma).

Western Blot Analysis of Hsps. HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10%

fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded into dishes 24 h prior to the treatment. Wine samples or the solvent (DMSO) as control was diluted with fresh culture medium as indicated in the figure legends, applied to the cells (~70% confluent), and incubated at 37 °C as shown.

Following the treatment, the cells were washed once with ice-cold phosphate-buffered saline, scraped, and collected. Lysis was followed in the lysis buffer [50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM DTT, and 1 mM PMSF] for 20 min on ice. The lysate was centrifuged at 13000*g* for 10 min at 4 °C in a microcentrifuge, and the supernatant was collected. Protein concentration was measured by using a protein assay kit (Bio-Rad). Equal amounts of cell extracts (10−30 μg) were denatured by boiling in Laemmli's sample buffer for 5 min, subjected to SDS-PAGE using a 10% running gel, and electroblotted to a nitrocellulose membrane (Hybond ECL). Nonspecific binding was blocked by incubation of the membrane with 10 mM Tris-HCl (pH 8.0) and 150 mM NaCl (TBS) containing 5% skim milk and 0.05% Tween 20 (TBS-T) for 2 h. The membrane was then incubated with a 1:1000 dilution of anti-Hsp70 (SPA-810, StressGen) or anti-Hsp27 (SPA-800, StressGen) in TBS-T containing 5% milk for 2−3 h. The membrane was then washed three times for 10 min each in TBS-T and incubated with a 1:5000 dilution of anti-mouse immunoglobulin peroxidase conjugate (Amersham) in TBS-T containing 5% milk for 1 h. After three washings for 10 min each in TBS-T, detection was performed using SuperSignal Western blotting detection reagents (Pierce) according to the manufacturer's protocol. The blots were scanned in a densitometry scanner (Sebia), and the intensity of the bands corresponding to the proteins being assayed was quantified. The band intensity in cells treated with the solvent (DMSO) was taken as 1.0. Data reported are from a representative experiment repeated two times with similar results.

Cell Culture and Cell Proliferation Assays. Dishes, media, and recombinant growth factors were described earlier (*19*, *33*). Bovine brain capillary endothelial cells (BBCE) were maintained in DMEM with low glucose concentration (1.0 mg/mL), 10% newborn calf serum, glutamine (2 mM), and antibiotics. Cultures received bFGF (2.5 ng/mL) every other day until confluent.

HeLa cells were maintained in DMEM with a high glucose concentration (4.5 mg/mL), 10% fetal calf serum, glutamine (2 mM), and antibiotics.

Cell proliferation assays were carried out as previously described (*19*, *33*). Briefly, stock cultures of BBCE cells were adjusted to a density of 5×10^3/mL and seeded in 1-mL aliquots into 12-well cluster dishes. After 16 h, wells received bFGF and either 5 μL aliquots of DMSO (control) only or wine samples in DMSO, and the same treatment was renewed after 48 h. Cells were counted with a Coulter particle counter when control wells were just confluent. HeLa cells were seeded at a density of 1×10^4/mL, and the assay was carried out exactly in the same way except that bFGF was not used. Values of cell densities represent the means of duplicate determinations, which varied by <10% of the mean.

RESULTS

Effect of Wine Total Extracts on Hsp Levels and Cell Populations in Vitro. All five red wines exhibited much higher total phenolics than the seven white ones, as expected. Syrah wine exhibited the highest content of total phenolics of ~3200 mg/L gallic acid equivalents (GAE), whereas these levels in Xinomavro and Limnio were 2700−2800 mg/L GAE. Agiorgitiko exhibited total phenolics of ~2100 mg/L GAE, whereas Mavrodaphne contained relatively low total phenolics of ~1770 mg/L GAE. All white wines exhibited similar total phenolics of 210−280 mg/L GAE.

In this set of experiments, we did not adjust the phenolic concentration in the wine total extracts used because our choice was to maintain the phenolic concentration of samples proportional to their concentration in the original wine.

The effect of total ethanol-free wine extracts on Hsp levels was tested in a human tumor cell line, namely, HeLa. We examined the inducible Hsp70, a member of the Hsp70 family, and the Hsp27, a member of the small Hsps family.

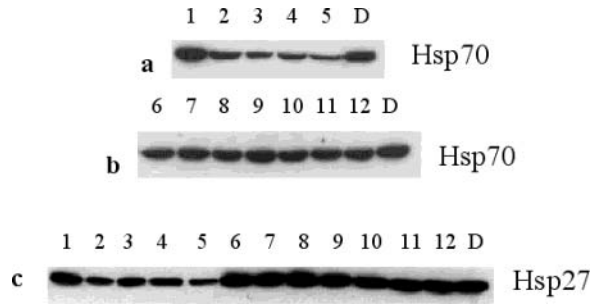

Figure 1. Effect of total wine extracts on Hsp70 and Hsp27 levels. Cells were treated with total ethanol-free extracts of red (lanes 1–5) or white (lanes 6–12) wines or only the solvent DMSO (D). The extracts were diluted 20-fold (panels **a** and **b**) or 50-fold (panel **c**) into the culture medium. After treatment for 5 h (**a**, **b**) or 24 h (**c**) at 37 °C, cells were lysed and analyzed by Western blotting using anti-Hsp70 (**a**, **b**) or anti-Hsp27 (**c**) antibodies. Lanes 1–12, total ethanol-free wine extracts: D = DMSO 1 = Mavrodaphne, 2 = Xinomavro, 3 = Agiorgitiko, 4 = Limnio, 5 = Syrah, 6 = Debina, 7 = Roditis, 8 = Athiri, 9 = Muscat, 10 = Robola, 11 = Sauvignon blanc, 12 = Traminer. Band intensities as quantified in a densitometry scanner were (of panel **a**) 1 = 1.1, 2 = 0.6, 3 = 0.4, 4 = 0.5, 5 = 0.3, D = 1.0; (of panel **b**) 6 = 0.9, 7 = 1.0, 8 = 1.1, 9 = 1.1, 10 = 1.0, 11 = 1.0, 12 = 0.9, D = 1.0; and (of panel **c**) 1 = 1.0, 2 = 0.5, 3 = 0.6, 4 = 0.5, 5 = 0.3, 6 = 1.1, 7 = 1.1, 8 = 1.2, 9 = 1.1, 10 = 1.0, 11 = 1.0, 12 = 1.0, D = 1.0. Total phenolic contents of the extracts used were 1 = 17200, 2 = 27950, 3 = 20820, 4 = 27040, 5 = 31840, 6 = 2600, 7 = 2360, 8 = 2140, 9 = 2360, 10 = 2580, 11 = 2760, and 12 = 2760 mg/L GAE.

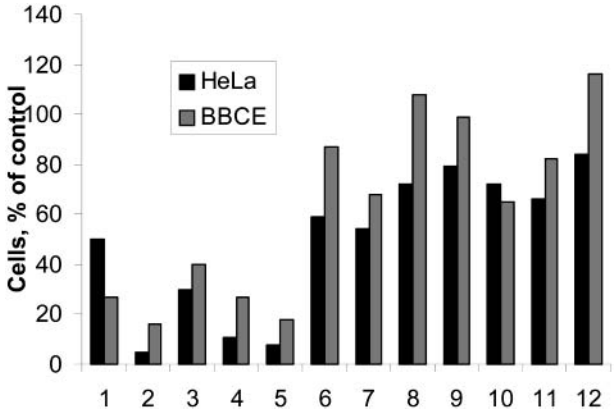

Figure 2. Decrease of the number of tumor (HeLa) and endothelial (BBCE) cells in cultures by total wine extracts: 1–12, total ethanol-free wine extracts; 1 = Mavrodaphne, 2 = Xinomavro, 3 = Aghiorgitiko, 4 = Limnio, 5 = Syrah, 6 = Debina, 7 = Roditis, 8 = Athiri, 9 = Muscat, 10 = Robola, 11 = Sauvignon Blanc, 12 = Traminer. Total phenolic contents of the extracts used were 1 = 3440, 2 = 5590, 3 = 4164, 4 = 5408, 5 = 6368, 6 = 2600, 7 = 2360, 8 = 2140, 9 = 2360, 10 = 2580, 11 = 2760, and 12 = 2760 mg/L GAE.

Four of five red wine extracts reduced significantly Hsp70 levels (**Figure 1a**). Among them, the most active was Syrah wine extract (**Figure 1a**), which exhibited the highest content of total phenolics and decreased Hsp70 levels ~3-fold. The nonactive red wine extract, that is, Mavrodaphne, contained the lowest level of phenolics, and it was the only sweet wine tested (**Figure 1a**).

Unlike red, white wine extracts contained much lower total phenolics and did not exhibit any effect on the level of Hsp70 (**Figure 1b**).

Table 1. Main Phenolics of Xinomavro (X) and Roditis (R) Wine Fractions and Subfractions

wine sample	main phenolics	wine sample	main phenolics
R1	phenolic acids, one flavone, one unclassified compound with max abs at 230 nm	X1	anthocyanins, flavanols, phenolic acids, flavonols
R2	phenolic acids, tyrosol	X2	flavanols, flavonols, phenolic acids, tyrosol, one unclassified compound with max abs at 280 nm
R3	phenolic acids	X3	phenolic acids, flavonols
R1a	phenolic acids, one flavone, one unclassified compound with max abs at 230 nm	X1a	flavanols, anthocyanins, flavonols, unclassified compounds with max abs at 230 nm, phenolic acids
R1b	NU	X1b	flavanols, one flavanone
R2a	phenolic acids, flavonols, tyrosol	X2a	flavanols, flavonols, phenolic acids, tyrosol, one unclassified compound with max abs at 280 nm, one flavone
R2b	flavonols, unclassified compounds with max abs at 280 nm, resveratrol	X2b	flavanols, flavonols, resveratrol, one unclassified compound with max abs at 280 nm
R3a	phenolic acids, one unclassified compound with max abs at 230 nm	X3a	phenolic acids, flavonols
R3b	NU	X3b	flavanols

[a] Not used.

Essentially the same results were obtained when the expression of Hsp27 was investigated (**Figure 1c**).

Both red and white wine extracts used contained total phenolics ~10 times higher than that of the original wines. This indicates that at total phenolic levels proportional to that of the original wines, red wine total extracts are more active in decreasing Hsp levels than white wine ones.

The effect of total ethanol-free wine extracts on tumor and endothelial cell population is presented in **Figure 2**. All red wine extracts decreased significantly the number of tumor and endothelial cells compared to controls. Among them, Xinomavro and Syrah wines appeared to be the most active, the cell numbers of these extracts being <10% of controls. On the other hand, white wine extracts exhibited a minor effect on HeLa cell numbers. Similarly, all red wine extracts decreased significantly the number of endothelial cells, whereas the effect of white wine extracts on endothelial cell numbers was considerably less prominent.

The red wine extracts used contained total phenolics ~2 times higher and the white wine extracts ~10 times higher than that of the respective original wines. This indicates that the ability of red wine total extracts to decrease tumor and endothelial cell numbers is much higher than that of white wine ones.

The present results suggest that red wine total extracts may significantly decrease Hsp levels and tumor and endothelial cell numbers, possibly due to their phenolic compounds. In the assays, total extracts used were diluted many times into the culture medium, that is, 20 or 50 times for Hsps and 200 times for cell proliferation. This indicates that total phenolics of red wine lower than those of the original wine may exhibit these biological activities.

Effect of Wine Phenolic Fractions on Hsp Levels and Cell Populations in Vitro. We then fractionated one red wine, Xinomavro, and one white wine, Roditis. As expected, Xinomavro and Roditis wines exhibited significant differences in total phenolic content and also in phenolic composition. Xinomavro wine exhibited 3380 mg/L GAE, containing mainly anthocyanins, flavanols, flavonols, and phenolic acids. On the contrary, Roditis wine exhibited 280 mg/L GAE, containing mainly phenolic acids and flavones. Both wines were fractionated into three fractions each, as described above. Each fraction was then subfractionated into nonpolymeric (subfraction a) and polymeric (subfraction b) phenolics. The main phenolic peaks of each fraction and subfraction determined by HPLC-DAD are shown in **Table 1**.

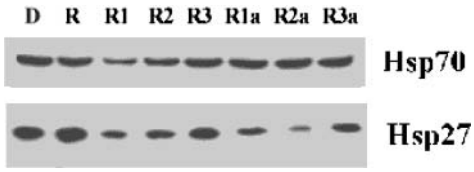

Figure 3. Effect of Roditis wine samples on Hsp70 and Hsp27 levels. The wine total extract (R), fractions (R1, R2, R3) and subfractions (R1a, R2a, R3a), or DMSO (D) was diluted 10-fold into the culture medium and applied to cells for 3 h at 37 °C. The cells were then lysed and analyzed by Western blotting using anti-Hsp70 (top) and anti-Hsp27 (bottom) antibodies. Band intensities as quantified in a densitometry scanner were (top) D = 1.0, R = 1.0, R1 = 0.7, R2 = 0.9, R3 = 1.1, R1a = 1.1, R2a = 1.1, and R3a = 1.1 and (bottom) D = 1.0, R = 1.0, R1 = 0.5, R2 = 0.6, R3 = 0.8, R1a = 0.5, R2a = 0.3, and R3a = 0.6. Total phenolic contents of the samples used were R = 5700, R1 = 5700, R2 = 7600, R3 = 5700, R1a = 3800, R2a = 5700, and R3a = 3800 mg/L GAE.

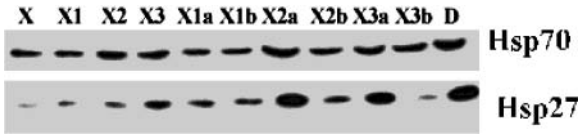

Figure 4. Effect of Xinomavro wine samples on Hsp70 and Hsp27 levels. The wine total extract (X), fractions (X1, X2, X3) and subfractions (X1a, X1b, X2a, X2b, X3a, X3b), or DMSO (D) was diluted 10-fold into the culture medium and applied to cells for 3 h at 37 °C. The cells were then lysed and analyzed by Western blotting using anti-Hsp70 (top) and anti-Hsp27 (bottom) antibodies. Band intensities as measured in a densitometry scanner were (top) X = 0.7, X1 = 0.7, X2 = 1.0, X3 = 1.0, X1a = 0.6, X1b = 0.6, X2a = 1.0, X2b = 0.7, X3a = 0.9, X3b = 0.8, and D = 1.0 and (bottom) X = 0.2, X1 = 0.3, X2 = 0.4, X3 = 0.8, X1a = 0.6, X1b = 0.6, X2a = 1.1, X2b = 0.7, X3a = 1.0, X3b = 0.4, and D = 1.0. Total phenolic contents of the samples used were X = 7600, X1 = 7600, X2 = 7600, X3 = 5700, and X1a, X1b, X2a, X2b, X3a, and X3b = 5700 mg/L GAE.

In this set of experiments, we used phenolic fractions of similar total phenolic content in order to evaluate the capacity of red versus white wine phenolics.

The effect of white and red wine total extracts, fractions, and subfractions on Hsp70 and Hsp27 levels is presented in **Figures 3** and **4**, respectively.

With regard to the Roditis white wine samples, their effects were predominantly on Hsp27 (**Figure 3**). Among Roditis fractions, R1 decreased Hsp27 levels by ~2-fold, and it also had an effect on Hsp70 levels. Moreover, the R2 fraction

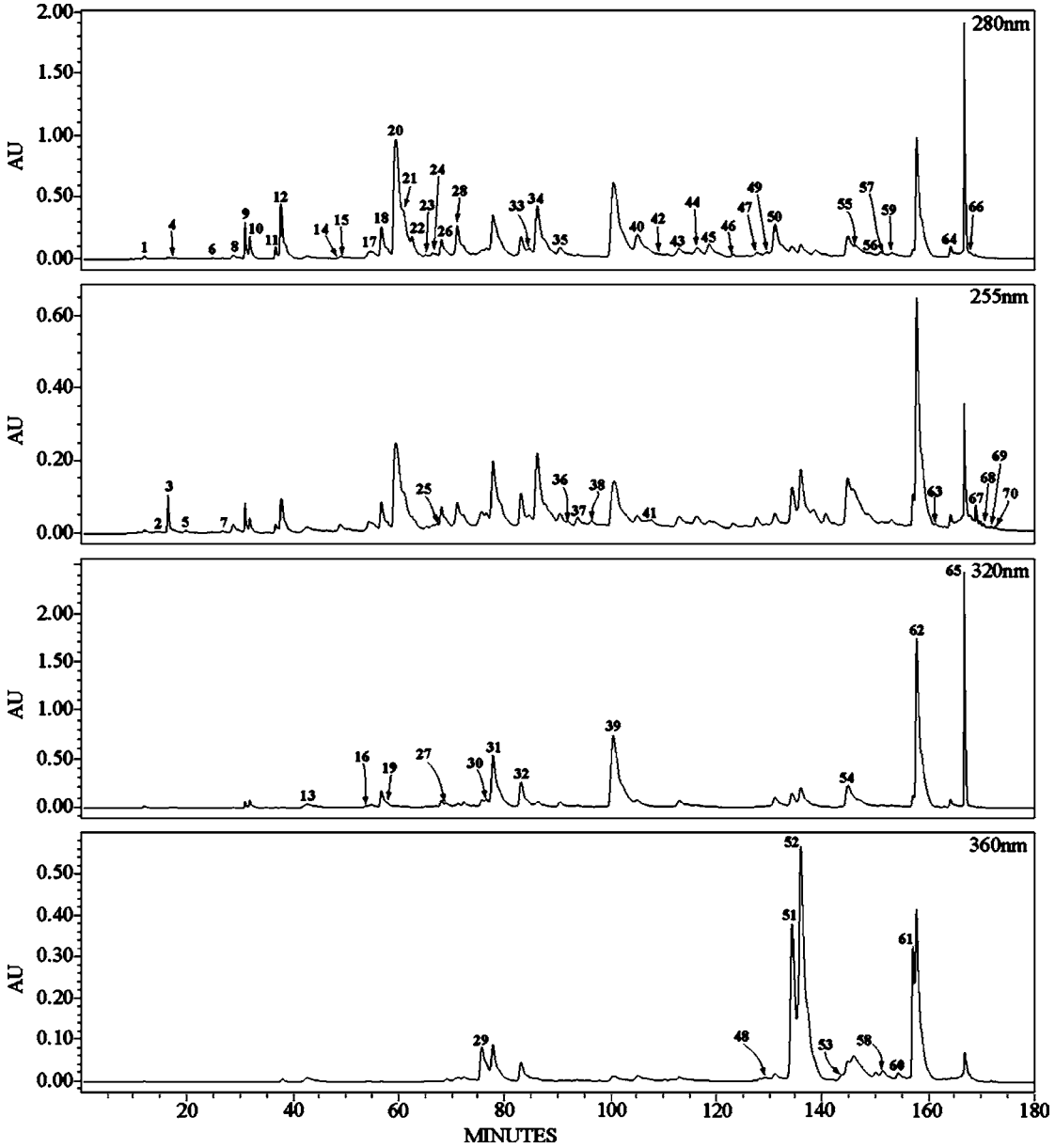

Figure 5. HPLC chromatograms of R2a subfraction at 280, 255, 320, and 360 nm. Peaks: 1, hydroxycinnamic acid; 2–5, benzoic acids; 6, flavanol; 7, benzoic acid; 8, gallic acid; 9, benzoic acid; 10–11, unclassifieds 280 nm; 12, flavanol; 13, hydroxycinnamic acid; 14, unclassified 280 nm; 15, benzoic acid; 16–17, unclassifieds 280 nm; 18, protocatechuic acid; 19, hydroxycinnamic acid; 20, tyrosol; 21–22, flavanols; 23, unclassified 280 nm; 24, catechin; 25, flavone; 26, flavanone; 27, hydroxycinnamic acid; 28, flavanol; 29, flavonol; 30, hydroxycinnamic acid; 31, caffeic acid; 32–33, hydroxycinnamic acids; 34, benzoic acid; 35, unclassified 280 nm; 36–37, hydroxycinnamic acids; 38, unclassified 230 nm; 39, *p*-coumaric acid; 40, unclassified 280 nm; 41, benzoic acid; 42, unclassified 280 nm; 43, flavanone; 44, flavanol; 45, unclassified 280 nm; 46, flavanol; 47, benzoic acid; 48, flavonol; 49–50, unclassifieds 280 nm; 51–53, flavonols; 54, hydroxycinnamic acid; 55–56, flavanones; 57, unclassified 280 nm; 58, flavone; 59, unclassified 280 nm; 60–61, flavonols; 62, hydroxycinnamic acid; 63, flavanone; 64–65, hydroxycinnamic acids; 66, flavanol; 67, unclassified 230 nm; 68, flavanol; 69, flavonol; 70, flavanol.

decreased considerably Hsp27 levels. The three subfractions containing nonpolymeric phenolics were also examined. Among them, the R2a subfraction reduced Hsp27 levels by ~3-fold, whereas the two others had also a significant effect (**Figure 3**). HPLC-DAD analysis of the R2a subfraction (**Figure 5**) revealed that it contained mainly hydroxycinnamic acids, benzoic acids, flavonols, and tyrosol, a well-known biophenol (*34*). All subfractions tested had no effect on Hsp70 levels.

Similarly to white wine, samples of Xinomavro red wine affected mainly Hsp27 levels (**Figure 4**). Among Xinomavro fractions, X1 decreased Hsp27 levels by ~3-fold, and it also had an effect on Hsp70 levels. Moreover, the X2 fraction strongly reduced Hsp27 levels.

With regard to subfractions, X3b strongly reduced Hsp27 levels. Moreover, subfractions X1a and X1b considerably reduced both Hsp27 and Hsp70 levels (**Figure 4**). The most active subfractions, that is, X3b, X1a, and X1b, were rich in flavanols, indicating that possibly such phenolic compounds may be very active.

The effect of total extracts, fractions, and subfractions was also tested on tumor and endothelial cell populations.

For Roditis white wine, fraction R2 exhibited the strongest inhibitory activity on tumor cells. Both R2a and R2b subfrac-

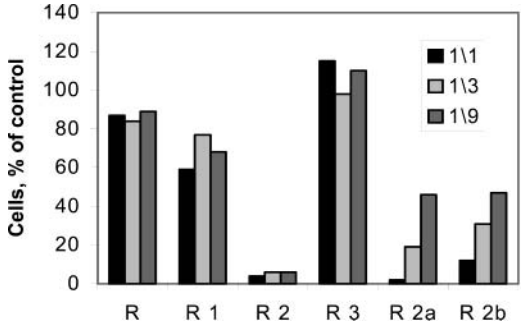

Figure 6. Decrease of the number of tumor (HeLa) cells in cultures by Roditis wine total extract (R), fractions (R1, R2, R3), and subfractions (R2a, R2b): 1/1, undiluted; 1/3, 3-fold dilution; 1/9, 9-fold dilution. Total phenolic contents of the samples used were R = 5700, R1 = 5700, R2 = 7600, R3 = 5700, R2a = 5700, and R2b = 3800 mg/L GAE.

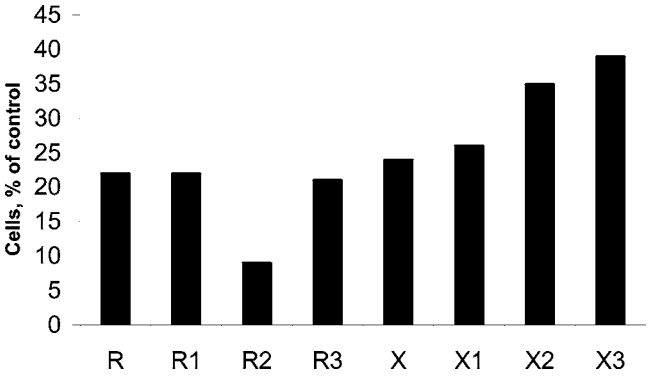

Figure 7. Decrease of the number of endothelial (BBCE) cells in cultures by Roditis and Xinomavro wines total extract (R, X) and fractions (R1, R2, R3, X1, X2, X3). Total phenolic contents of the samples used were R = 5700, R1 = 5700, R2 = 7600, R3 = 5700, X = 7600, X1 = 7600, X2 = 7600, and X3 = 5700 mg/L GAE.

tions were almost equally active (**Figure 6**). As mentioned above, the main peaks of R2a were of phenolic acids, flavonols, and tyrosol, whereas R2b contained mainly flavonols, unclassified compounds with maximum absorbance at 280 nm, and resveratrol. It should be noted that R2a and R2b contained tyrosol and resveratrol, respectively, two well-known biophenols (*1*, *21*, *34*). Fraction R2 showed also the strongest inhibitory activity on endothelial cell proliferation (**Figure 7**).

As far as Xinomavro red wine is concerned, the strongest inhibitory effect on tumor cell populations was exhibited by the X1 fraction and its X1b subfraction (**Figure 8**). X1b contained polymeric flavanols and one flavanone, indicating the potency of such polymeric phenolics. The different fractions of Xinomavro wine appeared to have only a minor effect on endothelial cell populations (**Figure 7**).

DISCUSSION

The results of the present study show that wine phenolic extracts—especially from red wines—decrease Hsp levels in culture cells. This new biological property adds to the multitude of activities exerted by wines. Indeed, red wine phenolic extracts rich in polymeric flavanols appear to decrease Hsp levels in a tumor cell line. Moreover, white wine phenolics may also result in a decrease of Hsp levels. Recently, it became clear that Hsp70 and Hsp27 protect cells from apoptotic cell death (*12*, *14*). On that basis, the decrease of these protein levels in tumor cells by wine phenolics may facilitate tumor cell death and thus contribute to the potent anticarcinogenic action of wine phenolics.

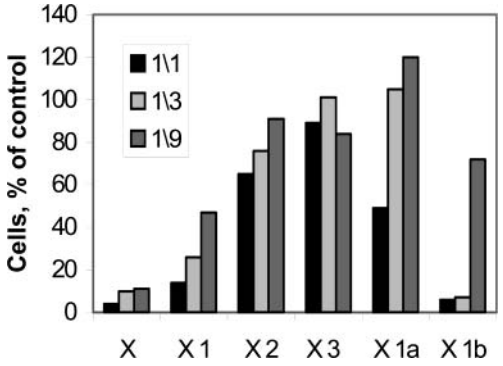

Figure 8. Decrease of the number of tumor (HeLa) cells in cultures by Xinomavro wine total extract (X), fractions (X1, X2, X3), and subfractions (X1a, X1b): 1/1, undiluted; 1/3, 3-fold dilution; 1/9, 9-fold dilution. Total phenolic contents of the samples used were X = 7600, X1 = 7600, X2 = 7600, X3 = 5700, X1a = 5700, and X1b = 5700 mg/L GAE.

As a consequence, wine phenolics should be further investigated to define the single active components and the potency of each. There is increasing pharmacological interest in the identification and characterization of compounds modulating Hsps. Such compounds may facilitate tumor cell death and could be further tested in diseases related to abnormal expression of Hsps such as cancer (*35*). There are only few natural flavonoids and only one synthetic compound that have been described to inhibit the induction of Hsp in tumor cell lines (*5*, *6*, *15*). In particular, the flavonols, quercetin and kaempferol as well as flavone, strongly inhibited Hsp expression, whereas luteolin, another flavone, exhibited only slight inhibition (*5*). Quercetin, for instance, inhibits the induction of Hsps at the transcriptional level, possibly through inhibition of heat shock factor 1 activation (*6*, *36*). Our results indicate that phenolics other than flavonols and flavones such as flavanols could also inhibit Hsp expression.

Considering the anticarcinogenic effect of wines, our results indicate that red and white phenolic extracts are active in decreasing tumor cell populations, in vitro. In agreement with this, a number of wine phenolics have been recently reported to inhibit the proliferation of human cancer cells (*2*, *4*, *37*). Indeed, catechin, epicatechin, quercetin, and resveratrol, which account for >70% of the total phenolics in red wine, have been shown to inhibit growth of human breast cancer cells at picomolar concentrations (*2*). The same compounds have been shown to potently inhibit prostate cancer cells (*4*). Furthermore, several plant flavonoids have been described to have antimitotic properties (*19*). Genistein, for instance, a dietarily ingested isoflavonoid, is a potent inhibitor of cell proliferation (*38*).

An interesting finding of the present study is that wine phenolics decrease endothelial cell populations, in vitro. Endothelial cell proliferation is a key event during angiogenesis, a process required by tumors to grow in size and metastasize. Toward this end, a number of dietarily derived flavonoids have been reported to inhibit endothelial cell proliferation and angiogenesis, in vitro (*19*). However, resveratrol is the only wine phenolic that has been reported to suppress endothelial cell proliferation (*1*, *3*).

In conclusion, our data show that wine phenolic preparations of red and, to a lesser degree, white wines can decrease heat shock protein levels in tumor cells and decrease tumor and endothelial cell populations. These results, taken together, indicate that wine phenolic extracts may affect cancer incidence by several independent mechanisms. Indeed, wine phenolic extracts have been shown to inhibit tumorigenesis in animal

models (*39*−*42*). Elucidation of the single active components responsible for the anticancer activity of wines is warranted. It will not only provide enhanced information regarding the active ingredients of our diet but will also possibly provide useful core molecules for chemical modifications with the long-term aim of developing pharmacologically potent anticancer agents.

ABBREVIATIONS USED

Hsps, heat shock proteins; HPLC-DAD, high-performance liquid chromatography diode array detector; DMEM, Dulbecco's modified Eagle medium; BBCE, bovine brain capillary endothelial; GAE, gallic acid equivalent.

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