Anticarcinogenic activity of polyphenolic extracts from grape stems against breast, colon, renal and thyroid cancer cells


A major part of the wineries’ wastes is composed of grape stems which are discarded mainly in open fields and cause environmental problems due mainly to their high polyphenolic content. The grape stem extracts’ use as a source of high added value polyphenols presents great interest because this combines a profitable venture with environmental protection close to wine-producing zones. In the present study, at first, the Total Polyphenolic Content (TPC) and the polyphenolic composition of grape stem extracts from four different Greek Vitis vinifera varieties were determined by HPLC methods. Afterwards, the grape stem extracts were examined for their ability to inhibit growth of colon (HT29), breast (MCF-7 and MDA-MB-23), renal (786-0 and Caki-1) and thyroid (K1) cancer cells. The cancer cells were exposed to the extracts for 72 h and the effects on cell growth were evaluated using the SRB assay. The results indicated that all extracts inhibited cell proliferation, with IC_{50} values of 121–230 μg/ml (MCF-7), 121–184 μg/ml (MDA-MB-23), 175–309 μg/ml (HT29), 159–314 μg/ml (K1), 180–225 μg/ml (786-0) and 134–400 μg/ml (Caki-1). This is the first study presenting the inhibitory activity of grape stem extracts against growth of colon, breast, renal and thyroid cancer cells.

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of limited economical interest (Arvanitoyannis et al., 2006), while the majority, along with other wastes of the wine making process, is discarded in nearby open fields. The environmental problems faced introduce the need for the development of method for their proper management via utilization.

Interestingly, one of our previous studies has shown that grape stem extracts are rich in bioactive polyphenols such as flavonoids, stilbenes and phenolic acids, suggesting their potential use as a source of high added value polyphenols, an activity that would combine the profitable venture with the environmental protection in wine-producing zones (Anastasiadi et al., 2009). Moreover, we have recently shown that grape stem extracts display significant antioxidant activity, protective activity against ROS-induced DNA damage and inhibitory activity against human hepatocellular and cervical cancer cells (Apostolou et al., 2014).

Having in mind the well established anticancer properties of grape stem extracts against liver and cervical cancer cells, this endeavor aims to extend these studies in order to investigate the inhibitory effects of the extracts against the growth of additional human cancer cell types such as breast, renal, thyroid and colon.

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid, (+)-catechin, (−)-epicatechin, p-coumaric acid, ferulic acid, caffeic acid, syringic acid, kaempferol, quercetin, rutin and trans-resveratrol were purchased from Sigma–Aldrich (Steinheim, Germany). The Folin–Ciocalteu reagent was chased from Sigma–Aldrich (Steinheim, Germany). The Folin–Ciocalteu reagent was purchased from Fluka (Steinheim, Germany). All solvents used for the qualitative and quantitative determination of polyphenols were purchased from J. T. Baker (Griesheim, Germany) as analytical (polyphenol extraction) or HPLC (chromatographic analyses) grades. All remaining chemicals were of analytical grade and obtained from Sigma–Aldrich.

2.2. Grapes and vinification byproduct

The samples studied were grape stems obtained from red (Violetomato, Mavrotragano, Hambourg Muscat) and white (Assyrtiko) varieties of V. vinifera species that are cultivated in Greece. All samples were obtained from Santorini island (Assyrtiko, Violetomato, and Mavrotragano) and Thessaly (Hambourg Muscat) during the 2009 and 2011 harvests. Stems samples were directly obtained by manual separation from the grape berries and were consecutively were air-dried, millpowdered and stored at room temperature.

2.3. Preparation of extracts

Fifty grams of dried sample stems were poured into a 200 ml mixture of methanol (MeOH)/H₂O/1.0N HCl (90:9.5:0.5 v/v) and sonicated in an ultrasonic bath for 10 min. The solution was separated by centrifugation and the remaining solid was re-extracted with the same solvent system and procedure. The combined extracts were evaporated under vacuum to provide a slurry, which was dissolved in 30 ml of MeOH:H₂O (1:1) and centrifuged for 10 min (7000 rpm). The supernatant liquid was extracted with petroleum ether (3 ml × 30 ml) in order to remove the contained 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). Thus, all sugars contained were separated in the aqueous layer and discarded. The combined organic layers were dried over anhydrous magnesium sulfate and evaporated under vacuum. The remaining solid was weighed and dissolved in MeOH to 1 mg/ml, filtered (0.45 μm) and subjected to HPLC analysis. In order to avoid the polyphenols degradation, all the aforementioned activities were performed in the absence of direct sunlight and at temperatures below 35 °C.

2.4. HPLC analyses

All HPLC analyses were carried out on a Hewlett Packard HP1100 system equipped with a quaternary pump and degasser. The column used was a Kromasil C18 column (250 mm × 4.6 mm, particle size 5 μm) connected with a guard column of the same material (8 mm × 4 mm). Injection was by means of a Rheodyne injection valve (model 7725i) with a 20 μl fixed loop. For the chromatographic analyses HPLC-grade water was prepared using a Milli-Q system, whereas all HPLC solvents were filtered prior to use through cellulose acetate membranes of 0.45 μm pore size. Chromatographic data were acquired and processed using Chromatstation software. The HPLC method used is a modified version of the method developed by Tsao and Yang (2003). More specifically, the analysis was carried out at 30 °C (maintained by a column thermostat) using samples of 20 μl, which were directly injected by means of a Rheodyne injection valve (model 7725i). The gradient eluted consisted of solvent A (obtained by the addition of 3% acetic acid in 2 mM sodium acetate aqueous solution) and solvent B (acetonitrile, CH₃CN). Run time was set at 70 min with a constant flow rate at 1.0 ml/min in accordance with the following gradient time table: at zero time, 95% A and 5% B; after 45 min, the pumps were adjusted to 85% A and 15% B; at 60 min, 65% A and 35% B; at 65 min, 50% A and 50% B; and finally at 70 min, 100% B. This routine was followed by a 30 min equilibration period with the zero time mixture prior to injection of the next sample. The analysis was monitored at 280, 320, and 360 nm simultaneously. Three replicate experiments were carried out for each sample examined. Peaks were identified by comparing their retention time and UV–vis spectra with the reference compounds, and data were quantitated using the corresponding curves of the reference compounds as standards. All standards were dissolved in methanol.

2.5. Assessment of the Total Phenolic Content (TPC)

The TPC of the extracts was determined in accordance with a modified version of the Folin−Ciocalteu method (Singleton et al., 1999). A 100 μl sample of extract was added to a 10 ml flask containing 6 ml of deionized water. One milliliter of Folin−Ciocalteu reagent was added to the mixture, and the flask was stopped and allowed to stand at room temperature for 3 min. A 1.5 ml portion of 20% Na₂CO₃ was added and the solution was diluted to the desire volume (10 ml) with deionized water. Absorbance was measured at 725 nm versus a blank after 2 h at room temperature. The results are expressed as gallic acid equivalents using the standard curve (absorbance versus concentration) prepared from authentic gallic acid.

2.6. Cell lines and culture maintenance

Human cancer cell lines used as targets were MCF-7 (breast, hormone dependent, ER positive), MDA-MB-231 (breast, hormone independent, ER negative), Caki-1 (kidney carcinoma), 786-O (renal adenocarcinoma), K1 (throid carcinoma) and HT29 (colon). All cells were cultured from the American Type Culture Collection (ATCC, Rockville, MD, USA) and the Imperial Cancer Research Fund (ICRF), London. Cells were routinely grown as monolayer cells in T-75 flasks (Costar) in an atmosphere containing 5% CO₂ in air, and 100% relative humidity at 37 °C. The culture medium used was Dulbecco’s modified Eagle’s medium, DMEM (Gibco, Glasgow, UK), supplemented with 10% fetal bovine serum (Gibco, Glasgow, UK), 2 mM glutamine (Sigma), 10% fetal bovine serum (Gibco, Glasgow, UK), supplemented with 10% fetal bovine serum (Gibco, Glasgow, UK), 2 mM glutamine (Sigma), 100 μg/ml streptomycin and 100 μg/ml penicillin. Cell passages were carried out by detaching adherent cells at a logarithmic growth phase by splitting the 2 × 3 ml of cell growth medium (Costar) – 0.02% trypan Blue (Cell culture incubation for 2–5 min at 37 °C. The loss of membrane integrity, as a morphological characteristic for cell death, was assayd by Trypan Blue exclusion (Gorman et al., 1996). The number of cells that were alive was estimated through a haemacytometer and phase-contrast microscopy. Each result represented the mean of four independent measurements and used for the inoculation of cells in the microplates. All chemicals and solvents used were of high purity and purchased from Sigma or Merck.

2.7. SRB cell proliferation assay

The stems extracts investigated were dissolved in DMSO to obtain a stock solution concentration of 40 mg/ml. The latter was further dissolved into DMSO to a final concentration less than 0.1%, which corresponds to a concentration that exhibits no effect on cell growth and proliferation, as was experimentally confirmed. Stock solutions were sterilized via filtration (0.22 μm) and stored at 24 °C. A constant concentration of solution each extract (800 and 600 μg/ml) was prepared in complete growth medium and used to make serial dilutions immediately after the extract was dissolved. All extracts were tested in nine graduated sextuplicate dilutions in complete growth medium, starting with a peak concentration of 400 μg/ml. The cytotoxic activity of all agents was tested in concentrations covering the range of 12.5–400 μg/ml. For the experiments, cells were plated (100 μl per well) in 96-well flat-bottom microplates (Costar–Conning, Cambridge) at various cell inoculation densities (MCF-7, MDA-MB-231, Caki-1, 786-O and K1: 8000 cells/well and HT29: 10,000 cells/well) so that untreated cells were in exponential growth phase at the time of cytotoxicity evaluation. Cells were left for 24 h at 37 °C to resume exponential growth and stabilization and afterwards exposed to tested agents for 72 h by the addition of an equal volume (100 μl) of either complete culture medium (control wells) or twice the final drug concentrations diluted in complete culture medium (test wells). Drug cytotoxicity was measured by means of SRB colorimetric assay estimating the survival fractions (SF) as the percent of control (untreated cells) absorbance. The SRB assay was carried out as previously described (Skehan et al., 1990) as modified by our group (Papazisi et al., 1997). In brief, culture medium was aspirated prior to fixation using a microplate-multiparticle wash device (Tri-Continental Scientific, Inc., Grass Valley, CA) and 50 μl of 10% cold (4 °C) trichloroacetic acid (TCA) were gently added to the wells. Microplates were left for 30 min at 4 °C. Washed five times with deionized water and left at room temperature for at least 24. Subsequently, 70 μl of 0.4% (w/v) sulfosalicylic acid solution were added to each well and left at room temperature for 20 min. SRB was removed and the plates were washed five times with 1% acetic acid before air drying. Bound SRB was solubilized with 200 μl
of 10 mM unbuffered Tris-base solution and plates were left on a plate shaker for at least 10 min. Absorbance was read in a 96-well plate reader at 492 nm subtracting the background measurement at 620 nm. The test optical density (OD) value was defined as the absorbance of each individual well, minus the blank value (“blank” is the mean optical density of the background control wells, n = 8). Mean values and the coefficient of variation (CV) from six replicate wells were calculated automatically.

Results were expressed as the “survival fraction” (SF), as shown below.

\[
\text{SF} = \frac{\text{mean OD of experimental wells}}{\text{mean OD of background control wells}} \times 100
\]

For each tested compound a dose–effect curve was produced. Sextuplicate determinations gave a CV (standard deviation/mean %) in the range of 1–5%, resulting in standard error (SE) which was very low in all cases. The data showing the mean optical density of the background control wells, n = 8). Mean values and the coefficient of variation (CV) from six replicate wells were calculated automatically.

Results were expressed as the “survival fraction” (SF), as shown below.

\[
\text{SF} = \frac{\text{mean OD of experimental wells}}{\text{mean OD of background control wells}} \times 100
\]

3. Results and discussion

The results of the assessment of the Total Polyphenolic Content (TPC) and the polyphenolic composition of the investigated extracts are presented in Table 1. The TPC and polyphenolic composition of grape stem extract of Hambourg Muscat (2009) variety have already been published (Apostolou et al., 2014), but are included herein for the reader’s convenience. The measured TPC values ranged from 318 to 415 mg GAE/g dry weight (dw) (Table 1) and are similar to those reported previously (Anastasiadi et al., 2009; Apostolou et al., 2014). Nevertheless, the highest TPC content was determined in stems extract of Mavrotragano (2011) variety. In addition to the polyphenolic composition, the presence of various flavanols, flavonols, phenolic acids and stilbenes was determined (Table 1). In particular, gallic (from 8.38 to 42.29 mg/g dried extract) and syringic acids (from 0.80 to 32.23 mg/g dried extract) were found to constitute the most abundant phenolic acids, while (+)-catechin (from 7.35 to 12.18 mg/g dried extract) and (-)‐epicatechin (from 4.51 to 19.13 mg/g dried extract) were the prevailing flavanols. Among flavonols, the molecules of quercetin (from 3.94 to 10.24 mg/g dried extract) and rutin (from 4.47 to 41.83 mg/g dried extract) were the most abundant, while the most intriguing finding was the large amounts of trans-resveratrol, a naturally occurring stilbene that is produced by a wide variety of plants in response to stress, injury, ultraviolet (UV) irradiation and fungal (e.g. Botrytis cinerea) infection. During the last decade there are numerous research results suggesting that, besides the antioxidant activity, trans-resveratrol displays potent anticancer (chemopreventive and therapeutic) activities (Aggarwal et al., 2004). The latter is associated with the ability of the molecule to suppress the proliferation of a wide variety of tumor cells, including lymphoid and myeloid cancers, multiple myeloma, breast, prostate, stomach, colon, pancreas and thyroid cancers, melanoma, head and neck squamous cell carcinoma and ovarian and cervical carcinoma (Liu et al., 2007).

Cell survival was estimated after 72 h of exposure to increasing concentrations of the tested extracts by means of the SRB assay. Taking into account that the tested agents were plant extracts, the in vitro cytotoxic activities of the extracts seem very promising. Our results revealed that all administered extracts caused a dose-dependent inhibition of cell proliferation. A 50% growth-inhibitory effect (IC50) was calculated for all extracts and the respective values are shown in Table 2. Representative microphotographs derived of various cell lines exposed to a series of Vodomato concentrations for 72 h are shown in Fig. 1. The dose–effect plots for all treated cell lines 72 h after the administration of the extracts are demonstrated in Fig. 2, where the different sensitivities of respective cell lines on the action of tested extracts are clearly shown. Moreover, the dose–effect plots indicate that the cytotoxic effect of the extracts, except to Mavrotagano’s effects on Caki-1 cells, was strictly concentration-dependent. As depicted in Fig. 2 and Table 2, the breast cancer cell lines, MCF-7 and MDA-MB-23, were revealed to be the most sensitive to all tested extracts, with IC50 values ranging between 121 and 230 μg/ml and 120 and 184 μg/ml, respectively. The most active extract displaying the lowest IC50 value was found to be from the Vodomato variety, the exception being the kidney and renal carcinoma cell lines (Caki-1 and 786-O, respectively).

The observed cell line chemosensitivity order was revealed to be the following: MDA-MB-231 ≈ MCF-7 > Caki-1 > K1 > HT29 > 786-O for the Vodomato variety extract, MDA-MB-231 > 786-O ≈ MCF-7 > HT29 > K1 ≈ Caki-1 for the Mavrotagano variety extract, Caki-1 ≈ MCF-7 ≈ MDA-MB-231 > 786-O > HT29 = K1, for the Assyrtilko variety extract and MCF-7 > MDA-MB-231 > K1 ≈ 786-O > HT29 = Caki-1, for the Muscat variety extract. Extracts from Mavrotagano and Assyrtilko varieties appeared to be equipotent against K1 and 786-O cancer cells, while those from Vodomato and Mavrotagano varieties had almost the same inhibitory effect against MDA-MB-231 breast cancer cells. On the contrary, the Mavrotagano variety extract had almost no inhibitory effect up to the maximum concentration used (400 μg/ml) against Caki-1 kidney carcinoma cells. Caki-1 cells were found to be the next more resistant cell line, exhibiting IC50 value for the Muscat variety extract slightly above the maximum concentration used (the survival fraction for 400 μg/ml dose was 0.533). For most

Table 1

<table>
<thead>
<tr>
<th>Grape variety</th>
<th>White variety</th>
<th>Red varieties</th>
<th></th>
<th></th>
<th></th>
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<tr>
<td>Gallic acid</td>
<td>8.38</td>
<td>11.48</td>
<td>42.29</td>
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<td>(+)-Catechin</td>
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<td>12.18</td>
<td>9.10</td>
<td>9.33</td>
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<tr>
<td>(-)-Epicatechin</td>
<td>15.23</td>
<td>19.13</td>
<td>4.51</td>
<td>13.32</td>
<td></td>
</tr>
<tr>
<td>trans-Resveratrol</td>
<td>2.15</td>
<td>9.09</td>
<td>25.41</td>
<td>15.32</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>7.54</td>
<td>3.94</td>
<td>10.24</td>
<td>8.21</td>
<td></td>
</tr>
<tr>
<td>Kaempferol</td>
<td>1.04</td>
<td>0.74</td>
<td>4.08</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.31</td>
<td>0.54</td>
<td>1.78</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Syringic acid</td>
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<td>17.44</td>
<td>2.85</td>
<td>32.23</td>
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<tr>
<td>Coumaric acid</td>
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<td>0.75</td>
<td>1.55</td>
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</tr>
<tr>
<td>Ferulic acid</td>
<td>0.31</td>
<td>3.59</td>
<td>0.59</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Rutin</td>
<td>16.00</td>
<td>15.93</td>
<td>4.47</td>
<td>41.83</td>
<td></td>
</tr>
<tr>
<td>TPC</td>
<td>372</td>
<td>415</td>
<td>318</td>
<td>407</td>
<td></td>
</tr>
</tbody>
</table>

All values are mg/g dried extract.

a TPC: Total Polyphenolic Content as mg gallic acid/g dried extract.

b Harvest year.

c The TPC and polyphenolic composition of stem extract from Muscat (or Moshato) 2009 was first published in Stagos et al. (2012).
Table 2
Cytotoxicity evaluation of the administered extracts expressed in IC50 values as determined based on SRB assay.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>MCF-7 IC50 (µg/ml)</th>
<th>MDA-MB-231 IC50 (µg/ml)</th>
<th>HT29 IC50 (µg/ml)</th>
<th>K1 IC50 (µg/ml)</th>
<th>786-O IC50 (µg/ml)</th>
<th>Caki-1 IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voidomato</td>
<td>121</td>
<td>120.5</td>
<td>175</td>
<td>159</td>
<td>200</td>
<td>134</td>
</tr>
<tr>
<td>Mavrotragano</td>
<td>230</td>
<td>124</td>
<td>260</td>
<td>300</td>
<td>225</td>
<td>&gt;400</td>
</tr>
<tr>
<td>Assyrtiko</td>
<td>180</td>
<td>184</td>
<td>309</td>
<td>314</td>
<td>225</td>
<td>100</td>
</tr>
<tr>
<td>Muscat</td>
<td>154</td>
<td>166</td>
<td>236</td>
<td>171</td>
<td>180</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

* IC50 values were derived from the corresponding dose-effect curves drawn from sextuplicate determinations with CV lower than 5%.

extracts, the most resistant cells demonstrated to be HT-29 and in a lesser degree Caki-1 and 786-O. Assyrtiko variety extract was found to exhibit better cytotoxic activity than the rest extracts, against Caki-1 cell line.

In colon cancer cells HT29, the IC50 values of the tested extracts ranged from 175 µg/ml (Voidomato variety) to 309 µg/ml (Assyrtiko variety). Previous studies have shown that grape seed extracts (GSE) inhibited several colon cancer cell lines such as LoVo, HT-29, SW480, Caco-2 and HCT-8 at concentrations from 25 to 100 µg/ml (Kaur et al., 2008; Dinicola et al., 2010, 2012). As regards the molecular mechanisms involved in the GSE-induced inhibition of colon cancer cell growth, it has been shown that GSE extracted from different cultures activate different apoptotic pathways, depending on the specific cancer cell line under treatment (Dinicola et al., 2012). In general, the molecular mechanisms of GSE-induced inhibition of colon cancer cell lines involve induction of cell cycle progression negative regulators (e.g. Cip1/p21 and Kip1/p27) and apoptosis through increases in caspases-9, -7 and -3 levels (i.e. by inducing mainly the intrinsic apoptotic pathway), apoptosis-inducing factor (AIF) and poly-ADP-ribose polymerase (PARP) levels (Dinicola et al., 2010; Kaur et al., 2008). Moreover, grape seed procyanidins have been shown to inhibit the growth of different colon cancer cell lines such as HT-29, SW-480 and LoVo, at concentrations from 25 to 50 µg/ml, through mainly induction in different cultures activate different apoptotic pathways, depending on the specific cancer cell line under treatment (Dinicola et al., 2012). In general, the molecular mechanisms of GSE-induced inhibition of colon cancer cell lines involve induction of cell cycle progression negative regulators (e.g. Cip1/p21 and Kip1/p27) and apoptosis through increases in caspases-9, -7 and -3 levels (i.e. by inducing mainly the intrinsic apoptotic pathway), apoptosis-inducing factor (AIF) and poly-ADP-ribose polymerase (PARP) levels (Dinicola et al., 2010; Kaur et al., 2008). Moreover, grape seed procyanidins have been shown to inhibit the growth of different colon cancer cell lines such as HT-29, SW-480 and LoVo, at concentrations from 25 to 50 µg/ml, through mainly induction in
apoptosis mediated by loss of mitochondrial membrane potential and by increased levels and activation of caspase-3 (Hsu et al., 2009). Interestingly, the most potent extract against HT29 colon cancer cells was from Voidomato variety which had also the highest content of gallic acid and trans-resveratrol among the tested grape stem extracts (Table 1) suggesting that these polyphenols may account for the observed inhibitory activity. Indeed, previous studies have shown that gallic acid (Yoshioka et al., 2000) and trans-resveratrol (Aires et al., 2013) inhibited colon cancer cell growth through apoptosis induction.

Two different cell lines, the hormone dependent MCF-7 and the hormone independent MDA-MB-231 were used for examining the inhibitory effects of grape stem extracts on breast cancer cell growth. All the tested extracts except to the Mavrotragano variety extract, exhibited similar IC50 values against the growth of both cell lines indicating that they exerted their effects through an estrogen receptor $\alpha$ (ER$\alpha$) independent mechanism. The Mavrotragano variety extract had almost a 2-fold less inhibitory potency against the ER$\alpha$ dependent MCF-7 cells. The only difference in the identified polyphenols between the Mavrotragano variety extract and the other extracts was that the former contained at least 6-fold more ferulic acid. Importantly, other studies have shown that ferulic acid induces MCF-7 cell growth through an estrogen dependent mechanism (Hao et al., 2010; Chang et al., 2006). Thus, it is inferred from the above that the polyphenolic composition of the grape stem extracts affects their inhibition against different breast cancer cell types.

Two different cell lines, 786-0 and Caki-1, were used to examine the potential protective effects of grape stem extracts against renal cell carcinoma. The Voidomato variety extract was the most potent extract with an IC50 value of 100 $\mu$g/ml against Caki-1 cell growth, while the Muscat variety extract was the most potent against 786-0 cell growth with an IC50 value of 180 $\mu$g/ml. In Caki-1 cells, the Mavrotragano variety exhibited an intriguing effect, since the growth inhibition was not dose-dependent but there was an increase in cell proliferation at about 200 $\mu$g/ml. An explanation for this observation may be that the grape stem extract is a mixture of different bioactive compounds. Some of these compounds inhibit cell growth, while others may act as mitogenic factors, and thus these compounds compete each other. Consequently, the concentration of 200 $\mu$g/ml may be a critical point at which the mitogenic factors prevail over the inhibitors of growth. Thus, similar to what was mentioned above for other cancer cell types, the results indicate that the inhibitory effects of the grape stem extracts depend on both the specific cancer cell line and the extract chemical composition. For example, Muscat variety extracts had higher amounts of syringic acid and rutin compared to other extracts suggesting that these polyphenolics may account for the high potency of the extracts against 786-0 cell growth. Both syringic acid (Kampa et al., 2004) and rutin (Yang et al., 2000) have been reported previously for anti-carcinogenic activity against different cancer types but not renal cell carcinoma. Moreover, this is the first study suggesting a potential inhibitory activity of grape extracts against renal cell carcinoma.

Grape stem extracts of Voidomato and Muscat varieties were the most potent against thyroid cancer cell K1 growth. This is the first study showing grape extract-induced inhibition against thyroid cancer cells. Remarkably, both Voidomato and Muscat variety extracts had higher content of trans-resveratrol compared to the other two extracts indicating that the high inhibitory potency of these extracts against K1 cell growth may be due to this polyphenolic. Indeed, trans-resveratrol has been shown previously to...
inhibit growth of different thyroid cancer cell lines (Shih et al., 2002; Kang et al., 2011).

In general, there are only few studies concerning the molecular mechanisms through which grape stem extracts inhibit cancer cell growth, although there are several studies with extracts from other parts of grapes (e.g. seeds and skin) (Kaur et al., 2009). Thus, it has been shown that grape seed extracts inhibited colorectal cancer cells through induction of apoptosis mediated by increase in pro-apoptotic proteins such as p21 and decrease in proteins inducing cell proliferation such as phosphatidylinositide 3-kinase (PI3Kinase) (Engelbrecht et al., 2007; Kaur et al., 2006). It has also been shown that grape seed extract inhibited both ligand epidermal growth factor (EGF)-induced and constitutively active EGF–Shc–ERK1/2–Elk1–AP1 pathway in human prostate cancer cells DU145 cells (Tyagi et al., 2003). Furthermore, grape seed inhibited the nuclear factor-kb (NF-kb) pathway and thus resulted in induction of apoptosis in DU145 cells (Dhanalakshmi et al., 2003).

Several studies have also investigated the molecular mechanisms through which the individual polyphenols found in the grape stem extracts exert their anticarcinogenic effects (Stagos et al., 2012). Thus, trans-resveratrol has been shown to inhibit thyroid cancer cells through activation of MAPK (ERK1/2), increase in p53 proteins and re-differentiation induction (Shih et al., 2002; Kang et al., 2011). Moreover, the anticancer activity of gallic acid has been shown to be due to ATM-Chk2 activation which leads to accumulation of cell division cycle 2 (cdc2) protein in its inactive form and consequently cell cycle arrest occurs (Zhou and Ellelode, 2000). Other mechanisms involved in anticancer activity of gallic acid are ribonucleotide reductase inhibition, the expression of this enzyme is significantly elevated in malignant tumor cells, cyclooxygenase-2 (COX-2) inhibition, playing significant role in inflammation and glutathione (GSH) depletion leading to oxidative stress and consequently to apoptosis (Verma et al., 2013). The molecular mechanisms proposed for the inhibitory effect of quercetin on cancer cell growth involve the inactivation of survival signal proteins as AKT, ERK, protein kinase C (PKC-alpha) as well as the activation of death signals as c-Jun N-terminal kinases (JNK) and PKC-delta (Granado-Serrano et al., 2008).

Moreover, the present results do not confirm that a high TPC is necessarily associated with a strong potency of the tested grape stem extracts against cancer cell growth. For example, although Mavrotragano and Muscat varieties extracts having the highest TPC exhibited the strongest inhibitory activity against cancer cell growth, the Voidomato variety extract with the lowest TPC had also the lowest IC50 values in five out of six cancer cell lines. We have also previously observed a lack of association between TPC and inhibitory activity of grape stem extracts against HepG2 and HeLa cancer cell growth (Apostolou et al., 2014). This lack of association could be attributed to the followings: (i) inhibitory activity against cancer cell growth is due also to other phytochemical compounds apart from polyphenols, (ii) there is a synergism between polyphenols and other phytochemical compounds and (iii) inhibitory activity is due to specific polyphenols present in the extracts. For example, it was noteworthy that the Voidomato variety extract, the most potent stem extract against all the tested cancer cell lines, except to 786-O, had especially high content of gallic acid and trans-resveratrol.

In conclusion, the aforementioned results reveal for the first time the anti-carcinogenic potentials of grape stems extracts against colon, breast, renal and thyroid cancers. Additionally, the ability of grape extracts to inhibit the growth of renal and thyroid cancer cells was also highlighted. Thus, the present results suggest that grape stem extracts may be beneficial for human health, since they may be used for prevention or even treatment against different cancer types. Moreover, grape stems constitute a major byproduct of the wine making process and their determined herein significant chemopreventive properties are indicative of their value as raw materials for the development of food supplements or ingredients for the preparation of high added value functional foods. In this respect further research is necessary for the determination of the molecular mechanisms involved in their anticancer activity as well as for the assessment of their cytotoxicity in normal cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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