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

Despina Sahpazidou[a,1], George D. Geromichalos[a,1], Dimitrios Stagos[b], Anna Apostolou[c], Serkos A. Haroutounian[c], Aristidis M. Tsatsakis[d], George N. Tzanakakis[e], A. Wallace Hayes[f,g], Dimitrios Kouretas[b,*]

[a] Cell Culture, Molecular Modelling and Drug Design Lab., Symeonidion Research Center, Theagenion Cancer Hospital, Thessaloniki 54639, Greece
[b] Department of Biochemistry and Biotechnology, University of Thessaly, Ploutonos 26 & Aiolou, Larissa 41221, Greece
[c] Chemistry Laboratory, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[d] Department of Forensic Sciences and Toxicology, Medical School, University of Crete, Heraklion 71003, Greece
[e] Laboratory of Anatomy-Histology-Embryology, Medical School, University of Crete, Heraklion 71003, Greece
[f] Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA
[g] Spherix Consulting, Inc., 6430 Rockledge Drive #503, Bethesda, MD 20817, USA

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ABSTRACT

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-MD-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.

1. Introduction

Vitis vinifera (grape) comprises one of the world's largest crops and along with its basic product, wine, has been part of the human diet since antiquity. Apart from their economic value, during the last years grape and wine were extensively studied due to their significant biological properties (Mullin, 2011). It must be pointed out that a broad variety of food supplements referred to as 'grape seed extracts' is consumed worldwide for their beneficial properties on human health. Our research group has demonstrated previously that grape extracts exhibit strong antioxidant activities and prevent the DNA damage induced by reactive oxygen species (ROS) (Stagos et al., 2005, 2006). Moreover, grape extracts have been reported to inhibit human cancer cell growth (Shrotriya et al., 2012) and tumor growth in various animal models (Sun et al., 2012). The molecular mechanisms involved in the anti-carcinogenic activities of grape extracts include the induction of apoptosis through modulation of cell signaling and cell-cycle regulators (Agarwal et al., 2000a), inhibition of enzymes playing an essential role in cell proliferation (e.g. human topoisomerase I) (Stagos et al., 2005) and inhibition of angiogenesis (Agarwal et al., 2004).

However, the wineries' wastes are harmful for the environment due to their high organic load and high acidity attributing mainly to their polyphenolic content. For this reason, most of the wine-producing countries as well as other institutions (e.g. European Union) have established regulations for the proper management of the winery's waste. A major part (3–5% of the processed grapes) of the solid wastes of wineries is composed of grape stems. Currently, a small portion of grape stems is used as animal feed and/or in the production of natural organic fertilizers (compost), processes

Abbreviations: AIF, apoptosis-inducing factor; PARP, poly-ADP-ribose polymerase; ROS, reactive oxygen species; SF, survival fractions; TCA, trichloroacetic acid; TPC, Total Polyphenolic Content.

* Corresponding author. Tel.: +30 2410565277; fax: +30 2410565293.
E-mail address: dkouret@uth.gr (D. Kouretas).

[1] The two authors have contributed equally to the present work.

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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 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 (Voidomato, Mavrotragano, Hambourg Muscat) and white (Assyrtiko) varieties of *V. vinifera* species that are cultivated in Greece. All samples were obtained from Santorini island (Assyrtiko, Voidomato, 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)/H_2O/1.0 N HCl (90:9.5:0.5 v/v) and sonicated in an ultrasonic bath for 10 min. The solvent was separated by filtration and the remaining solid was re-extracted three additional times, using 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/H_2O (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, membrane 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 Chemstation 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, CH_3CN). 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 stoppered and allowed to stand at room temperature for 3 min. A 1.5 ml portion of 20% Na_2CO_3 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 (thyroid carcinoma) and HT29 (colon). All cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and the Imperial Cancer Research Found (ICRF), London. Cells were routinely grown as monolayer cell cultures in T-75 flasks (Costar) in an atmosphere containing 5% CO_2 in air, and 100% relative humidity at 37 °C and sub-cultured twice a week, restricting the total number of cell passages below 20. 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), 100 μg/ml streptomycin and 100 IU/ml penicillin. Cell passages were carried out by detaching adherent cells at a logarithmic growth phase by addition of 2–3 ml of a 0.05% trypsin (Gibco) – 0.02% EDTA (Sigma) mixture and incubation for 2–5 min at 37 °C. The loss of membrane integrity, as a morphological characteristic for cell death, was assayed by Trypan Blue exclusion (Gorman et al., 1996). The number of cells that were alive was estimated through a haematocytometer 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 2–4 °C. A concentrated solution of 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-Corning, 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 (Papazisis et al., 1997). In brief, culture medium was aspirated prior to fixation using a microplate-multiwash device (Tri-Continent 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 to dry at room temperature for at least 24 h. Subsequently, 70 μl of 0.4% (w/v) sulforhodamine B (Sigma) in 1% acetic 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

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