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Effects of Grape Cell Culture Extracts on Human Topoisomerase II Catalytic Activity and Characterization of Active Fractions

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Grape and its cell culture extracts are rich in flavonoids and stilbenes that are biologically active. The objective of this study was to evaluate possible inhibitory effects of grape (a *Vitis* hybrid Bailey Alicant A) cell culture extract and subfractions on human DNA topoisomerase II catalytic activity and to characterize constituents in the most potent fractions. At 5 μg/mL, grape cell crude extract and Toyopearl (TP) fractions 2−6 provided significantly greater inhibition of topoisomerase II catalytic activity than quercetin, a chemopreventive agent previously known as a topoisomerase catalytic inhibitor. The most potent topoisomerase II catalytic inhibitors from grape cell culture extracts in descending order of potency were TP fractions 4 and 6 (IC_{50} = 0.28−0.29 μg/mL), TP-3 (IC_{50} = 0.74 μg/mL), and crude extract (IC_{50} = 1.02 μg/mL); each was significantly more potent than resveratrol (IC_{50} = 18.0 μg/mL), another well-known chemopreventive topoisomerase II catalytic inhibitor. Using both high-performance liquid chromatography and liquid chromatography electrospray ionization mass spectrometry, constituents in TP-4 and TP-6 were characterized. These constituents included cyanidin-3,5-diglucoside, malvidin-3-acetylglucoside, peonidin-3-coumaryl-5-diglucoside, procyanidin B_1, procyanidin B_2, procyanidin B_5, procyanidin dimer digallate, procyanidin C_1, myricetin, and rutin, none of which have been previously characterized from grape cell cultures. The significant potency especially of TP-4 and TP-6 from grape cell cultures suggests that these fractions may have potential as chemopreventive agents.

KEYWORDS: Human topoisomerase II; catalytic inhibitor; antitopoisomerase II activity; *Vitis*; grape cell culture; chemopreventive agents

INTRODUCTION

Cancer is the second largest cause of mortality in the United States, and the mortality rate has not been lowered since 1950 (*1*). Carcinogenesis consists of three distinct steps—initiation, promotion, and progression—that can be interfered with by a wide array of chemopreventive phytochemicals and their mixtures (*2*, *3*). Initiation, an irreversible event, begins when normal cells are exposed to a carcinogen and their cDNA undergoes damage, which remains unrepaired or misrepaired. Promotion is a process of active proliferation of damaged cells. Progression irreversibly produces a new clone of tumor cells that proliferate, invade, and spread to other tissues (metastasis).

Unlike the empirical approach used to screen and develop chemopreventive agents in the past, progress in molecular techniques allows a more mechanistic approach in the selection process (*4*). As an example, DNA topoisomerases have been target enzymes for new anticancer drug discovery. Topoisomerases are essential enzymes in cell proliferation in all living organisms since they are involved in DNA processes such as replication, transcription, translation, recombination, and chromosome dynamics, simply by regulating DNA topology. Type I topoisomerase is a monomeric enzyme that breaks one DNA strand to permit another DNA to get in. Type II topoisomerase is a dimeric and ATP-dependent enzyme that breaks both DNA strands at once, allowing the entry of another intact DNA helix (*5*).

Especially, topoisomerase II has emerged as a chemotherapeutic target for a diverse group of antitumor agents (*4*−*6*). Topoisomerase inhibitors constitute a class of chemopreventive agents that inhibit carcinogenesis via their antiproliferative or cell-differentiating action. Clinically useful chemotherapeutic drugs including etoposide (VP-16) and anthracyclines are topoisomerase II poisons. These poisons interrupt completion of topoisomerization by stabilizing the enzyme−DNA complex and stimulating the formation of cleavable complexes. Another class of topoisomerase II inhibitors is the catalytic inhibitors,

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Table 1. Retention Time, UV–Visible Absorption Spectra, and Molecular Weights of Phenolic HPLC Standards

HPLC standards	t_R,[a] HPLC (min)	HPLC-DAD[b] λ_{max} (nm)	molecular weight
gallic acid (GA)	7.2	270	170.1
(–)-gallocatechin (GC)	9.0	270	306.3
pelargonidin-3,5-diglucoside (Pg-3,5-diglc)	11.4	280, 330, 515	595.5[c]
delphinidin-3-glucoside (Dp-3-glc)	11.5	280, 345, 430sh, 525	465.2[c]
procyanidin B_1 (PB_1)	11.5	280	578.5
(–)-epigallocatechin (EGC)	12.5	280	306.3
cyanidin-3-galactoside (Cy-3-gal)	13.8	275, 332sh, 420sh, 500	484.9[c]
(+)-catechin hydrate (C)	15.0	280	290.3
cyanidin-3-glucoside (Cy-3-glc)	15.0	280, 330, 430sh, 515	449.2[c]
petunidin-3-glucoside (Pt-3-glc)	16.5	280, 345, 435sh, 525	479.2[c]
procyanidin B_2 (PB_2)	17.1	280	578.5
caffeic acid (CAF)	18.9	290sh, 320	180.2
pelargonidin-3-glucoside (Pg-3-glc)	19.0	275, 330, 428sh, 500	433.2[c]
(–)-epigallocatechin gallate (EGCG)	20.7	275	458.4
(–)-epicatechin (EC)	20.9	280	290.3
peonidin-3-glucoside (Pn-3-glc)	21.0	280, 325sh, 435sh, 515	463.2[c]
malvidin-3-glucoside (Mv-3-glc)	22.0	275, 345, 460sh, 525	493.2[c]
(–)-gallocatechin gallate (GCG)	23.4	275	458.4
trans-astringin (A)	24.2	300sh, 320	406.4[c]
ellagic acid hydrate (EA)	34.0	255, 305sh, 320	302.2
trans-piceid (Pic)	34.7	310	390.4[c]
(–)-epicatechin gallate (ECG)	35.2	275	442.4
rutin hydrate (R)	35.6	255, 295sh, 355	664.6
quercetin-4-glucoside (Q-4-glc)	49.6	255, 265sh, 365	449.4[c]
myricetin (M)	51.5	260sh, 300sh, 370	318.2
resveratrol (RV)	53.7	280, 310, 340sh	228.2
quercetin dihydrate (Q)	58.4	250, 305sh, 360, 395sh	338.3
kaempferol (K)	61.5	265, 295sh, 320sh, 365	286.2

[a] t_R, retention time in minutes. [b] DAD, diode array detector; sh, maximum of the shoulder in the spectrum. [c] Values were calculated by molecular weight calculation (www.chemie.fu-berlin.de/cgi-bin/molform).

which antagonize topoisomerase II on the DNA. Many chemopreventive agents are found to be topoisomerase II catalytic inhibitors, suggesting this as a useful strategy to select chemopreventive agents that may be effective at the stage of promotion and progression (*4*, *6*). Topoisomerase II is essential for cell division and cell proliferation since it is required for the completion of mitosis. While differentiated cells express very low levels of topoisomerase II, highly proliferative tumor cells often express 25−300 times higher levels than those of quiescent cells (*7*).

Grape and its cell culture extracts are rich in flavonoids and stilbenes that are biologically active (*8*−*10*). The widespread consumption of grapes throughout the world creates the possibility of exploiting the properties of their constituents (particularly polyphenols) as chemopreventive agents. For example, *trans*-resveratrol has been reported to have a chemopreventive activity against three major stages of carcinogenesis—initiation, promotion, and progression—through its antioxidant and antimutagenic activities (*8*). Cell culture systems, which accumulate less interfering compounds such as pectins, enzymes, and complex sugars than fruits, simplify the isolation and purification of bioactive compounds of complex forms including procyanidins (*11*, *12*).

The objective of this research was to evaluate possible inhibitory effects of grape (a *Vitis* hybrid Bailey Alicant A) cell culture extract and subfractions on human DNA topoisomerase II catalytic activity and to characterize the chemical profiles of the potent fractions.

MATERIALS AND METHODS

Biological Materials. Highly pigmented callus cultures of grape (a *Vitis* hybrid, Bailey Alicant A) were generously donated by Dr. M. Shuler (Department of Chemical Engineering, Cornell University, Ithaca, NY). The original callus cultures were established from *Vitis* hybrid [(*V. lincecumii* × *V. labrusca* × *V. vinifera*) × (*V. vinifera* × *V. vinifera*)] on solid Gamborg's B-5 medium (*13*, *14*).

Chemicals and Reagents. *High-Performance Liquid Chromatography (HPLC) Standards*. Anthocyanin mixture (3-glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin), *trans*-astringin, cyanidin-3-galactoside, pelargonidin-3,5-diglucoside, *trans*-piceid, and quercetin-4-glucoside were purchased from Polyphenols Laboratories (Sandnes, Norway). Myricetin was purchased from Fluka Chemical Corp. (Milwaukee, WI), and procyanidin B_1 and procyanidin B_2 were purchased from ChromaDex Inc. (Santa Ana, CA). Other HPLC standards such as caffeic acid, (+)-catechin hydrate, ellagic acid hydrate, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, gallic acid, (−)-gallocatechin, (−)-gallocatechin gallate, kaempferol, quercetin dihydrate, resveratrol, and rutin hydrate were purchased from Sigma Chemical Co. (St. Louis, MO).

Topoisomerase II Catalytic Activity. A topoisomerase drug screening kit and human DNA topoisomerase II were purchased from TopoGEN, Inc. (Columbus, OH). Certified molecular biology agarose was obtained from Bio-Rad (Hercules, CA). Dimethyl sulfoxide (DMSO), 10× Tris acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer, and other chemicals needed for this assay were obtained from Sigma Chemical Co.

Ferric Reducing Antioxidant Power (FRAP) Assay. 2,4,6-Tripyridyltriazine (TPTZ) and ferric chloride were purchased from Sigma Chemical Co. and Fisher Scientific Company (Fair Lawn, NJ), respectively.

Cell Culture Procedure. Callus cultures of grape were maintained by monthly subculture of 2 g segments to 50 mL of maintenance medium (MM) with 0.8% Phytagar (Gibco/BRL), contained in GA7 vessels (*14*). MM consists of Gamborg's B-5 macronutrients, micronutrients, and vitamins (*15*) supplemented with 2% (w/v) sucrose and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Prior to autoclaving at 121 °C for 18 min, the pH of MM was adjusted to 5.8.

Cell suspension cultures were initiated by inoculating 2 g of callus into 50 mL of MM without agar, contained in 250 mL flasks (*14*) at 150 rpm with 1 in. orbit. Cell suspensions were subcultured every other

week by transferring 10 mL of cell suspension culture including 2.5 mL of packed cell volume into 50 mL of MM. Both callus and suspension cultures were maintained under continuous fluorescent light (158 ± 2 μmol m^{-2} s^{-1}) at 25 °C.

Harvest and Extraction of Cells. Cell suspension cultures were harvested after 14 days using vacuum filtration through Whatman #4 filter paper until the point when liquid drops had ceased to exude for at least 30 s. The harvested cell cultures were measured for their fresh biomass (g/flask).

Cells were held at −80 °C until extraction to promote cell lysis and prevent degradation of phytochemicals. Harvested cells (54.1 g) were extracted six times with 70% (v/v) aqueous acetone (250 mL, 15 s each at high speed) using a Turbo-twister blender (Hamilton Beach/Proctor-Silex Inc., Southern Pines, NC). After cell debris was removed using vacuum filtration through Whatman #4 filter paper, the resultant extract was concentrated under vacuum using a rotary evaporator at 40 °C in order to remove acetone and some of the water. A 2 mL aliquot from the resulting 42 mL of viscous residue was lyophilized to measure DW (88.5 mg), and the lyophilized crude extract was stored at −20 °C for bioassay.

Fractionation of Cell Crude Extract. Vacuum liquid chromatography (VLC) was applied in this study in order to fractionate the cell culture crude extract. A profile of components of the most bioactive fractions of grape cell culture extract was characterized by using reversed phase HPLC and LC-electrospray ionization (ESI)/mass spectrometry (MS).

The remaining residue (40 mL) was fractionated by VLC on HW-40F Toyopearl (TP) resin polymer (column inner diameter × height, 6.5 cm × 4 cm) (TOSOH Bioscience LLC, Montgomeryville, PA). After the viscous residue was loaded, the column was washed with 100 mL of water. The first fraction (TP-1) was eluted with an additional 100 mL of water. The second to sixth fractions (TP fractions 2−6) were eluted with 50% aqueous methanol (250 mL), 100% methanol (250 mL), methanol−acetone (1:1, v/v, 250 mL), 100% acetone (250 mL), and 50% aqueous acetone (500 mL), respectively. Each fraction was concentrated under reduced pressure, frozen at −20 °C, and then lyophilized. The dry weights (DWs) of the first to sixth fractions were 299.2, 273.1, 354.0, 159.0, 16.2, and 100.8 mg, respectively, and the fractions were stored at −20 °C for bioassay.

HPLC Analysis of TP Fractions. The TP fractions were analyzed by HPLC using a Beckman system gold HPLC (Fullerton, CA) equipped with a pump 126 (Beckman) and a photodiode array detector 168 (Beckman). Standard and samples (40 μL) were injected onto a 4.6 mm × 250 mm C_{18} column (5 μm, Discovery C18, Supelco, United States) with a 4.0 mm × 20 mm C_{18} guard cartridge (5 μm, Discovery C18 Supelguard Cartridge, Supelco). The gradient elution was performed with solvent A, consisting of 0.4% formic acid (v/v) in water, and solvent B, comprising 0.4% formic acid in acetonitrile (v/v) at a flow of 0.7 mL/min as follows: 0−1 min, 5−10% B; 1−45 min, 10−20% B; 45−65 min, 20−60% B. Prior to each run, the column was washed for 15 min and equilibrated for at least 30 min. Peaks were detected at the retention times of standards along with spectrum confirmation. Also, groups of mixed internal standards were added to samples and ran in order to spike peaks for additional assurance.

LC-ESI/MS Analysis of the Most Potent TP Fractions. Analysis was carried out on a Q-TOF Ultima API mass spectrometer, equipped with 2795 separations module, 2996 photodiode array detector, and Masslynx software (Waters Corporation, Milford, MA). ESI/MS was operated under positive ion mode (and negative if necessary) at a range of *m*/*z* 100−1950. HPLC separation was performed as described above, except that the flow rate for ESI/MS analyses was only 0.07 mL/min due to postcolumn split (1:10).

Human Topoisomerase II Catalytic Activity. Several assays have been developed to evaluate the ability of a compound to modulate biochemical events presumed to be mechanistically linked to carcinogenesis (*16*). Examples of such assays include topoisomerase inhibitors, which constitute a class of chemopreventive agents that inhibit carcinogenesis via their antiproliferative or cell-differentiating action (*4*).

The topoisomerase II catalytic activity was monitored via electrophoresis using topoisomerase II drug screening kit (TopoGEN, Inc.). Briefly, 20 μL of reaction mixtures contained 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mM dithiothreitol. Supercoiled DNA (pRYG) provided in the kit was determined to be ideal for this assay since it contains a single, high affinity topoisomerase II cleavage and recognition site. After 1 μL (0.25 μg) of pRYG DNA was added, followed by the addition of 2 μL of test compounds in solvent (10% DMSO unless specified otherwise), the reaction was initiated by adding 4 units (2 μL) of human DNA topoisomerase II and carried out at 37 °C for 75 min. The reaction was terminated by adding 2 μL of 10% sodium dodecyl sulfate (SDS) followed by digestion with 0.6 μL of proteinase K (1.25 units/μL, 25 μg/μL) at 52 °C for 15 min to degrade enzyme. After 2 μL of loading buffer (0.25% bromophenol blue and 50% glycerol) was added to the mixture, DNA was extracted from the mixture by adding 20 μL of chloroform:isoamyl alcohol (24:1), followed by centrifugation at 10000*g* for 20 s. A portion (15 μL) of the upper blue layer containing extracted DNA was loaded onto 1% agarose gel. Electrophoresis was conducted at 66 V (2 V/cm) for 5 h in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) using Classic CSSU 2025 Electrophoretic Gel System, E-C Apparatus Corporation (Florida). Supercoiled DNA (pRYG) and relaxed DNA were included in the electrophoresis run as markers for DNA topology. Gels were then stained in 0.5 μg/mL ethidium bromide in distilled water for 30 min and destained for 15 min in distilled water prior to digital image acquisition using Kodak Image Station 440 CF. The optimized lane and band identification was analyzed using KODAK 1D Image Analysis Software version 3.5. The inhibitory activity was expressed as relative activity of topoisomerase enzyme in the presence of the test compound in comparison to that in the negative control solution. Solvent used in dissolving test compounds was added to the negative control solution to get an unbiased measurement of the effect of solvent on enzyme activity.

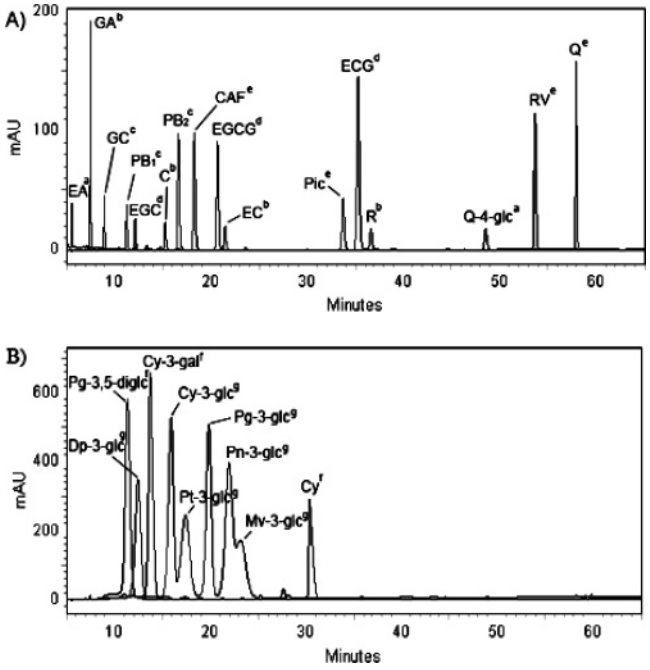

Figure 1. Superimposed HPLC chromatograms of seven groups of HPLC standard mixtures (a–g) detected at 280 nm. The mixtures of phenolic standards were used for internal standard spikes for additional assurance of peak identification. (**A**) Ellagic acid hydrate (EA), gallic acid (GA), (–)-gallocatechin (GC), procyanidin B_1 (PB_1), (–)-epigallocatechin (EGC), (+)-catechin hydrate (C), procyanidin B_2 (PB_2), caffeic acid (CAF), (–)-epigallocatechin gallate (EGCG), (–)-epicatechin (EC), *trans*-piceid (Pic), (–)-epicatechin gallate (ECG), rutin hydrate (R), quercetin-4-glucoside (Q-4-glc), resveratrol (RV), and quercetin dihydrate (Q). (**B**) Pelargonidin-3,5-diglucoside (Pg-3,5-diglc), delphinidin-3-glucoside (Dp-3-glc), cyanidin-3-galactoside (Cy-3-gal), cyanidin-3-glucoside (Cy-3-glc), petunidin-3-glucoside (Pt-3-glc), pelargonidin-3-glucoside (Pg-3-glc), peonidin-3-glucoside (Pn-3-glc), malvidin-3-glucoside (Mv-3-glc), and cyanidin (Cy).

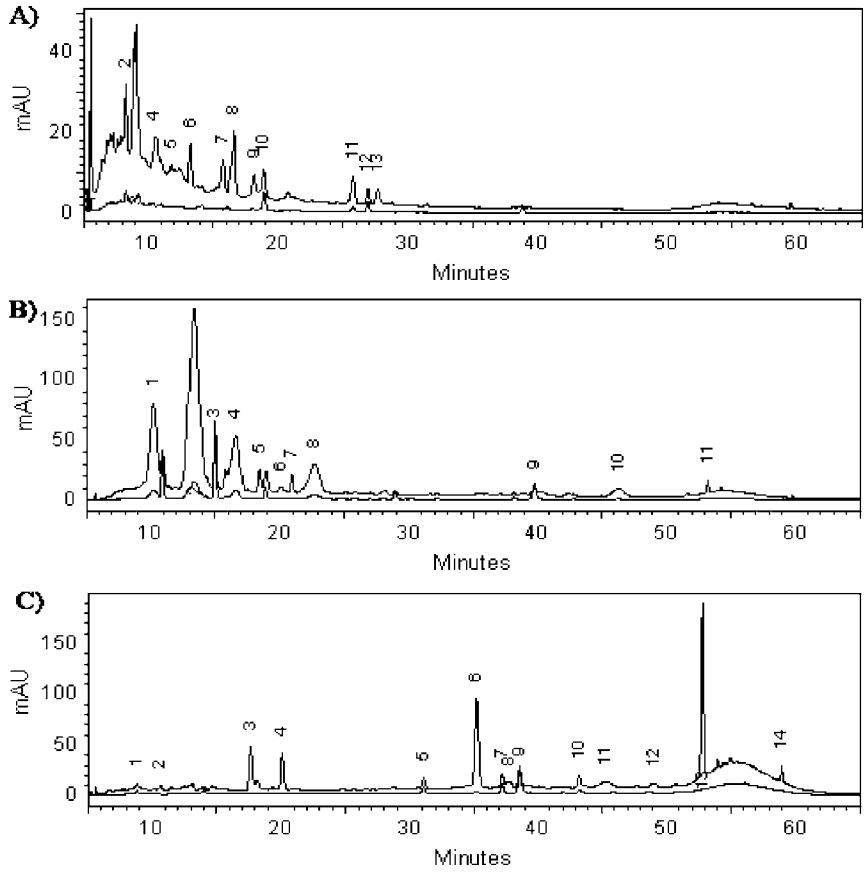

Figure 2. Typical HPLC chromatograms of (**A**) TP) fraction 1 [peak 3 (GC), peak 5 (EGC), peak 6 (C), and peak 8 (PB_2)], (**B**) TP fraction 2 [peak 2 (Dp-3-glc), peak 3 (C/ Pg-3,5-diglc), peak 4 (Cy-3-glc), and peak 8 (Pn-3-glc)], and (**C**) TP fraction 3 [peak 1 (GC)] from grape cell suspension culture were detected at 280 (upper baseline) and 365 (lower baseline) nm. See **Table 1** for abbreviations.

FRAP Assay. The antioxidant capacity of the grape cell crude extract and TP fractions was evaluated by the FRAP assay described by Benzie and Strain (*17*) with some modifications. The FRAP assay measures the ability of the antioxidants contained in a sample to reduce ferric−TPTZ (Fe^{3+}−TPTZ) complex to a ferrous (Fe^{2+}) form that absorbs light at 593 nm. Freshly prepared 95 μL of FRAP reagent ($FeCl_3 \cdot 6H_2O$ and TPTZ in acetate buffer) was mixed with 5 μL of diluted sample in clear 96 well plates (Fisher Scientific, Hanover Park, IL), and the absorbance was read at 593 nm after 10 min of reaction at 25 °C. The results were obtained by comparing the absorption change in the test compound with those obtained from increasing concentrations of quebracho tannin and expressed as mg of quebracho tannin equivalents (QTE) per mg of dry sample. Quebracho tannin, generously provided by Dr. D. Seigler (Department of Plant Biology, University of Illinois, Urbana−Champaign, IL), was used as a standard ($y = 0.5096x + 0.0326$, $r^2 = 0.9921$) at a range of final concentrations of 1.25−50 μg/mL.

Statistical Analyses. Significant differences between treatments were determined by the ANOVA test for analysis of variances followed by Fisher's least significant difference test at $\alpha = 0.05$ probability of type one comparisonwise error rate.

RESULTS AND DISCUSSION

Twenty-eight phenolic HPLC standards were used to identify peaks by retention time along with spectrum confirmation (**Table 1**). Chemical profiles of TP fractions 1−6 from grape cell suspension cultures were characterized by reversed phase HPLC. Peak identification of TP fractions 2, 4, and 6 was confirmed by internal standard spikes (**Figure 1**). Typical HPLC chromatograms from TP fractions 1−6 were characterized as follows (**Figures 2** and **3**). The most hydrophilic fraction, TP-1, included gallocatechin, epigallocatechin, catechin, and procyanidin B_2. The anthocyanin-rich fraction TP-2 contained delphinidin-3-glucoside, catechin, pelargonidin-3,5-diglucoside, cyanidin-3-glucoside, and peonidin-3-glucoside. Only gallocatechin was detected in TP-3 among all tested standards, and the dominant peaks 3, 4, and 6 showed their absorption maxima at 310−315 nm. TP-4 was rich in polyphenolic compounds such as procyanidin B_1, catechin, procyanidin B_2, piceid, epicatechin gallate, rutin, myricetin, and resveratrol. TP-5 contained epigallocatechin, catechin, epicatechin, myricetin, and resveratrol. Most constituents of TP-6 were coeluted between 51 and 60 min as a large unresolved peak that has been previously reported as a mixture of oligomers and polymers of procyanidins in grape seeds (*18*, *19*). The antitumor-promoting effects of grape seed polyphenols of various degrees of polymerized procyanidins were much more potent than green tea polyphenols of mostly monomers (*20*). Other constituents in TP-6 were procyanidin B_1 and resveratrol. Among 28 tested standards, six compounds have been reported to be topoisomerase II catalytic inhibitors, and four of these were found in TP fractions (shown in parentheses): procyanidin B_2 (TP-1 and TP-4), epicatechin gallate (TP-4), myricetin (TP-4 and TP-5), resveratrol (TP fractions 4−6), quercetin, and kaempferol (*4*, *21*, *22*). This is the first report of the presence of gallocatechin, epigallocatechin, procyanidin B_1, procyanidin B_2, myricetin, and rutin in grape cell cultures using HPLC.

For further characterization of unknown peaks from TP-4 and TP-6, which were the most potent antitopoisomerase II fractions (data shown later), LC-ESI/MS was performed under positive ion mode (and negative if necessary) at a range of *m*/*z* 100−1950. The major ions were singly charged. The summary

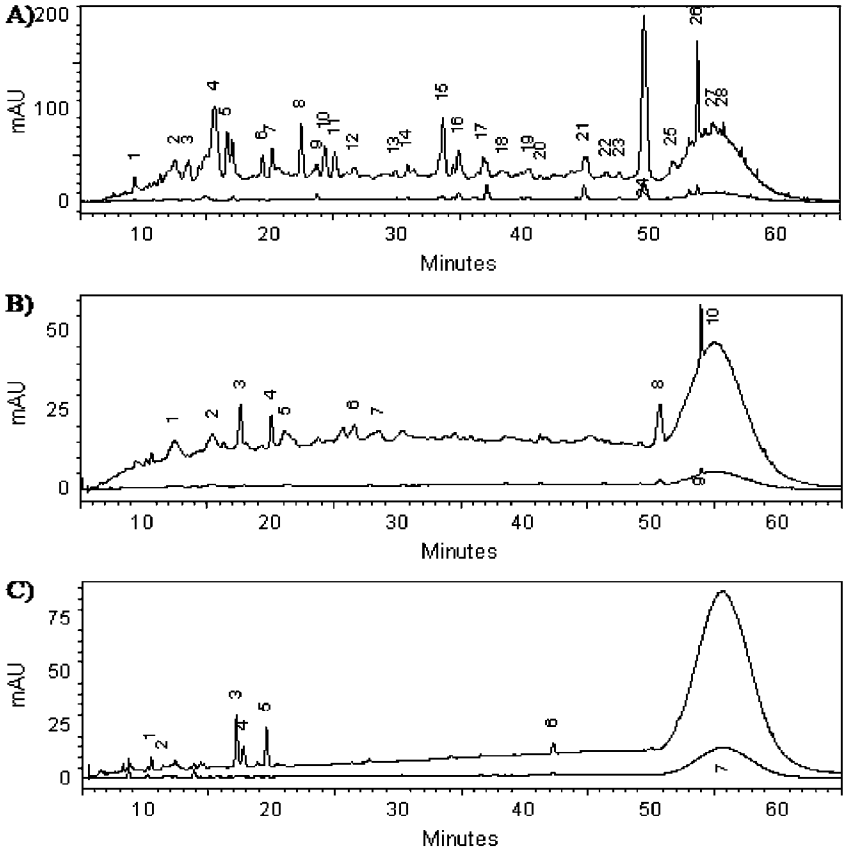

Figure 3. Typical HPLC chromatograms of (**A**) TP fraction 4 [peak 2 (PB_1), peak 4 (C), peak 5 (PB_2), peak 15 (Pic), peak 16 (ECG), peak 17 (R), peak 24 (M), and peak 26 (RV)], (**B**) TP fraction 5 [peak 1 (EGC), peak 2 (C), peak 5 (EC), peak 8 (M), and peak 9 (RV)], and (**C**) TP fraction 6 [peak 2 (PB_1) and peak 7 (RV)] from grape cell suspension culture were detected at 280 (upper baseline) and 365 (lower baseline) nm. See **Table 1** for abbreviations.

of the characterization of peaks from TP-4 and TP-6 is shown in **Tables 2** and **3**, respectively.

TP fraction 4 consisted of anthocyanins, procyanidins, stilbenes, and other phenolic compounds (**Table 2**). On the basis of molecular ion, spectrum, and retention time, cyanidin-3,5-diglucoside (*m*/*z* 611.1), peonidin-3,5-diglucoside (*m*/*z* 625.2), peonidin-3-coumaryl-5-diglucoside (*m*/*z* 771.2), cyanidin-3-coumarylglucoside (*m*/*z* 595.2), and peonidin-3-coumarylglucoside (*m*/*z* 609.2) were determined to be present in peaks 1, 2, 19, 24, and 26, respectively (*23*). More studies are needed to determine if peonidin-5 coumaryl-3-diglucoside is present; however, the evidence from the literature supports our preliminary identification of peonidin-3-coumaryl-5-diglucoside (*23*, *24*). Cyanidin-3,5-diglucoside and peonidin-3-coumaryl-5-diglucoside, found in grape juices (*23*), are newly reported for grape cell cultures in the present study. The identity of catechin in peak 4 was confirmed by molecular ion at *m*/*z* 289.0 detected under negative ion mode. The retention time of the molecular ion at *m*/*z* 291.1 (peak 7) was close to but different from (−)-epicatechin, and thus, peak 7 was assigned as an epicatechin isomer. The identity of (−)-epicatechin gallate in peak 16 was also supported by its molecular ion (*m*/*z* 442.3). (−)-Epicatechin gallate, also found in green tea and red wine, has been reported to be a chemopreventive agent (*25*, *26*). The identity of procyanidin B_1 (peak 2) and procyanidin B_2 (peak 5) was confirmed by their molecular ions (*m*/*z* 579.2). Other B type procyanidin dimers were found in peaks 4, 6, 8, 10, and 17, based on molecular ions (*m*/*z* 579.2) and spectra (*20*). Procyanidin trimers were found in peaks 3 and 8, based on molecular ions (*m*/*z* 867.2) and spectra, and the latter peak was identified as procyanidin C_1 (PC_1) by its retention time (*20*, *27*, *28*). As a trimer of epicatechins with 4−8 linkages, PC_1 was one of the most potent antioxidants found in grape seeds (*20*). PC_1 has not been previously reported in grape cell cultures. Procyanidin tetramers (*m*/*z* 1153.3) and pentamers (*m*/*z* 1441.3) were also found in peaks 10 and 11, respectively, based on molecular ions detected under negative ion mode and spectra (*18*, *19*). Preliminary characterization of peaks 12 and 13 as procyanidins was based on absorption maxima at 280 nm. Recently, the presence of procyanidin dimer (*m*/*z* 579.1), trimer (*m*/*z* 867.0), tetramer (*m*/*z* 1173.9), pentamer (*m*/*z* 1461.9), and hexamer (*m*/*z* 1752.9) was reported in grape cell cultures (*10*). These procyanidins have been previously found to have antioxidant and chemopreventive effects (*20*, *21*, *29*). Preliminary characterization of peak 9 as a flavonoid was based on its spectrum (*30*). A molecular ion at *m*/*z* 807.3 (peak 14) detected under the negative ion mode has been characterized as a product of polymerization between epicatechin and malvidin-3-glucoside by an ethyl bridge in grapes and wine (*31*, *32*). However, the presence of this compound in peak 14 was not supported by the spectral data. The signal of *m*/*z* 883.2 (peak 15) has been previously found in grape seeds and determined to be procyanidin dimer digallate based on molecular ion, retention time, and thiolysis degradation studies (*27*, *33*, *34*). Procyanidin dimer digallate has been previously reported to be a potent catalytic inhibitor of topoisomerase II (*21*). Both polymerized product between epicatechin and malvidin-3-glucoside and procyanidin dimer digallate have not been reported in grape cell cultures. The identity of *trans*-piceid in peak 15 was confirmed by molecular ion (*m*/*z* 389.1) detected under negative ion mode (*35*). The signal of *m*/*z* 243.1 in peak 16 was identified as piceatannol based on molecular ion and retention time (*35*). As a stilbene found in

Table 2. Chromatographic and Spectrophotometric Characteristics of Polyphenolic Compounds in TP Fraction 4 of Grape Cell Cultures

			LC-ESI/MS		
peak[a]	t_R,[b] HPLC	HPLC-DAD[c] λ_{max} (nm)	t_R	$[M + H]^+$, *m/z*[d]	identity[e]
1	9.5	280, 440sh, 525	9.6	737.2, 611.1	Cy-3,5-diglc (611.1)
2	12.7	280, 520	14.1	579.2, 625.2	PB_1 (579.2), Pn-3,5-diglc (625.2)
3	13.6	280	15.8	867.2	P_3[f] (867.2)
4	15.7	280	18.5	579.2, (289.0)	PB[g] (579.2), C (289.0)
5	16.9	280	19.5	579.2	PB_2
6	19.5	280, 310	22.2	579.2	PB (579.2)
7	20.4	280	23.8	291.1, 139.0	EC isomer (291.1)
8	22.5	280	27.2	867.2, 579.2	PC_1 (867.2), PB (579.2)
9	23.8	260, 285sh, 310sh	27.8	(403.1)	a flavonoid[h]
10	24.5	280	29.6	579.2, (1153.3)	PB (579.2), P_4[i] (1153.3)
11	25.2	280	30.8	(720.2, 1441.3)	P_5[j] (1441.3)
12	26.9	280	33.0	nd[k]	P[l]
13	30.0	280	35.1	(713.2)	P
14	30.9	270, 315sh	36.4	(807.3)	EC-ethyl-(Mv-3-glc)[m]
15	33.8	290–320	39.4	883.2, (389.1)	procyanidin dimer digallate (883.2), Pic (389.1)
16	35.0	260, 280, 344, 365	41.1	243.1, 442.3	piceatannol (243.1), ECG (442.3)
17	37.0	255, 280, 295sh, 355	43.2	579.2	PB (579.2), R
18	38.6	280, 315sh	44.6	nd	a flavanone
19	40.6	280, 325sh, 525	46.4	897.2, 771.2	Pn-3-coumaryl-5-diglc (771.2)
20	41.5	280, 315sh	48.4	nd	a flavanone
21	45.2	285, 315	51.6	(611.2)	ni[n]
22	46.9	280, 315sh	nd	nd	a flavanone
23	47.8	275, 315sh, 365	nd	nd	ni
24	49.8	280, 310sh, 520	53.4	595.2	Cy-3-coumarylglc (595.2), M
25	51.9	280, 315sh	nd	nd	a flavanone
26	53.9	280, 310sh, 520	54.9	609.2	Pn-3-coumarylglc (609.2), RV
27	55.3	280, 315sh	nd	nd	a flavanone
28	56.1	280, 315sh	nd	nd	a flavanone

[a] See **Figure 3A**. [b] t_R, retention time in minutes. [c] DAD, diode array detector; sh, maximum of the shoulder in the spectrum. [d] The major signals were given in the order of relative intensities. Also, the *m/z* values from negative ESI-MS are shown in parentheses. [e] The *m/z* values are shown in parentheses. For abbreviations, refer to **Table 1**. [f] Procyanidin trimer. [g] B type of procyanidin dimer. [h] Preliminary characterization. [i] Procyanidin tetramer. [j] Procyanidin pentamer. [k] Not detected. [l] Procyanidin. [m] Tentatively assigned as a polymerized compound between epicatechin and malvidin-3-glucoside by an ethyl bridge. [n] Not identified.

Table 3. Chromatographic and Spectrophotometric Characteristics of Polyphenolic Compounds in TP Fraction 6 of Grape Cell Cultures

			LC-ESI/MS		
peak[a]	t_R,[b] HPLC	HPLC-DAD[c] λ_{max} (nm)	t_R	$[M + H]^+$, *m/z*[d]	identity[e]
1	10.5	260sh, 290sh	11.2	625.2	Pn-3,5-diglc (625.2)[f]
2	11.5	280	nd[g]	nd	PB_1
3	17.3	290sh, 310	18.9	149.0	cinnamic acid isomer (149.0)
4	17.9	260sh, 290sh	nd	nd	ni[h]
5	19.7	290sh, 310	21.4	(265.0)	ni
6	42.4	280sh, 315sh	44.8	309.1, 149.0	cinnamic acid (149.0)
7	55.7	280, 315sh	55.9	243.1, 149.0, 228.0, 251.0, 552.4, 579.4, 535.3, 596.4, 667.4, 338.4, 684.5, 391.3, 728.5, 755.5	piceatannol (243.1), cinnamic acid (149.0), RV (228.0), RV-diglc (552.4), PB_5 (579.4), Mv-3-acetylglc (535.3), Cy-3-coumarylglc (596.4), *p*-coumaryl pigment A (755.5)[f]

[a] See **Figure 3C**. [b] t_R, retention time in minutes. [c] DAD, diode array detector; sh, maximum of the shoulder in the spectrum. [d] The major signals were given in the order of relative intensities. Also, the *m/z* values from negative ESI-MS are shown in parentheses. [e] The *m/z* values are shown in parentheses. For abbreviations, refer to **Table 1**. [f] Preliminary characterization. [g] Not detected. [h] Not identified.

grapes, wines, and grape cell cultures, *trans*-piceatannol (also named astringinin) has been reported to be the most potent antioxidant (two times more efficient than trolox) among different grape stilbenes and, thus, a potential chemopreventive agent (*9*, *36*). Molecular ion signals at peaks 18, 20, 22, 25, 27, and 28 were not strong enough to be characterized under both positive and negative ion modes. The preliminary assignment of each peak as a flavanone was based on absorption maxima (nm) at 280 and 315sh (*30*).

Table 3 presents characteristics of polyphenolic compounds in TP-6. Preliminary characterization of peak 1 as peonidin-3,5-diglucoside (*m/z* 625.2) was based on molecular ion and retention time (*23*) but not a UV−visible spectrum because the concentration was too low. The molecular ion signal (*m/z* 149) at peak 3 was likely to be cinnamic acid, but its retention time was not in good agreement with previous references, and thus, we assigned it as a cinnamic acid isomer (*37*). The molecular ion at *m/z* 149.0 of peak 6 was characterized as cinnamic acid, based on molecular ion and retention time (*37*, *38*). Cinnamic acid, present in wines (*37*), has been reported to have a chemopreventive activity (*39*).

Most constituents of TP-6 were coeluted as a large unresolved peak (peak 7 in **Figure 3C**). The LC-ESI/MS spectrum of TP-6 (data not shown) presents a major peak under the positive ion mode at a range of *m/z* 100−1950. The molecular ion at *m/z* 243.1 was identified as piceatannol based on molecular ion and retention time (*35*). Cinnamic acid (*m/z* 149.0) was also detected in this peak based on molecular ion and retention time (*37*, *38*). The identity of resveratrol in this peak was also supported by molecular ion at *m/z* 228.0. Preliminary characterization of the

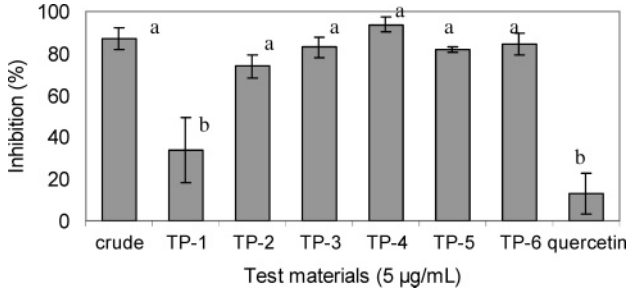

Figure 4. Inhibition of catalytic activity of human DNA topoisomerase II by grape cell crude extract and TP fractions as compared to quercetin at the final concentration of 5 μg/mL. Data are means ± SEM ($n = 4$). Values with different letters (a, b) are statistically different ($p < 0.0001$).

signal of *m*/*z* 552.4 as resveratrol-3,4′-diglucoside was based on molecular ion, although it was not likely that resveratrol diglucoside was eluted later than *trans*-piceid (also named *trans*-resveratrol-3-glucoside). Recently, *trans*- and *cis*-resveratrol-3,4′-diglucoside (*m*/*z* 553.2) were found in grape cell cultures in low concentrations (*26*). The signal of *m*/*z* 579.4 in the peak was determined to be procyanidin B_5 (PB_5), based on molecular ion and retention time (*20*, *27*). The presence of PB_5, found in grape seeds (*20*, *27*), has not been previously reported in grape cell cultures. The signals of *m*/*z* 535.3 and *m*/*z* 596.4 were assigned as malvidin-3-acetylglucoside and cyanidin-3-coumarylglucoside, respectively, based on their molecular ions and retention times (*10*, *23*). However, their visible spectra could not be observed in the coeluted peak, possibly due to their low concentrations. These anthocyanins have also been found in grape juices (*23*). The molecular ion at *m*/*z* 755.5 has been detected in wine and proposed as *p*-coumaryl pigment A (*40*), of which a visible spectrum was not observed in the present study due to its low concentration. Further fractionation of TP-4 and TP-6 is currently under way in our laboratory in order to achieve complete identification of bioactive constituents. The crude extracts from grape cell suspension culture and TP fractions were evaluated for their catalytic inhibitory activities on human DNA topoisomerase II using the DNA relaxation assay, which is a valuable in vitro test to select chemopreventive agents (*4*). The requirement of topoisomerase II for the completion of mitosis makes this enzyme essential for cell division and cell proliferation (*7*). The inhibition of topoisomerase II may contribute to the overall chemopreventive activity of grape cell culture extracts, making them promising candidates for the investigation of chemopreventive agents.

Resveratrol (a stilbene) and quercetin (a flavonol) were used as positive controls because they are present in grapes, and most of all, they are well-known chemopreventive agents and topoisomerase II catalytic inhibitors (*2*, *4*, *8*, *41*). As shown in **Figure 4**, the inhibition of catalytic activity of topoisomerase II by grape cell crude extracts and TP fractions 2−6 was significantly higher than quercetin at a fixed concentration of 5 μg/mL ($p < 0.0001$). TP-1, the most hydrophilic fraction, also showed an inhibitory activity comparable to quercetin at 5 μg/mL. Resveratrol did not show any inhibition of catalytic activity of topoisomerase II (data not shown) at the concentration tested (5 μg/mL); however, the activity significantly increased at a higher concentration (25 μg/mL). The potency of crude extracts from grape cell culture was found to be reproducible from three independent cell culture batches. The inhibition of catalytic activity of topoisomerase II by grape cell crude extract was not affected by storage at −20 °C for 1.5 years as a lyophilized powder. The significance of the data shown in **Figure 4** is that

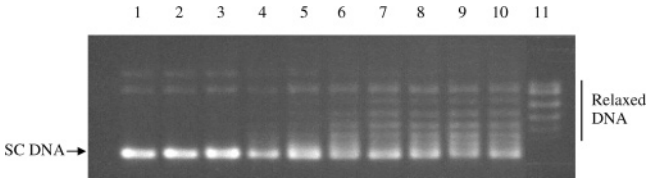

Figure 5. Representative relaxation assay for determining the inhibition of catalytic activity on human DNA topoisomerase II by TP fraction 4 from grape cell culture. Substrate supercoiled (SC) pRYG DNA (1 μL or 0.25 μg) was incubated with 4 units (2 μL) of human DNA topoisomerase II (lanes 2–10), and the concentrations of TP-4 are shown below: lane 1, SC pRYG DNA marker; lanes 2 and 3, 1 μg/mL; lanes 4 and 5, 0.5 μg/mL; lanes 6 and 7, 0.25 μg/mL; lanes 8 and 9, 0.05 μg/mL; lane 10, none (negative control); and lane 11, relaxed pRYG DNA marker.

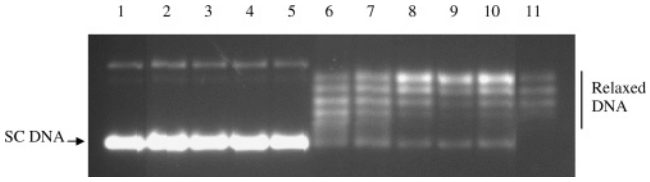

Figure 6. Representative relaxation assay for determining the inhibition of catalytic activity on human DNA topoisomerase II by TP fraction 6 from grape cell culture. Substrate supercoiled (SC) pRYG DNA (1 μL or 0.25 μg) was incubated with 4 units (2 μL) of human DNA topoisomerase II (lanes 2–10), and the concentrations of TP-6 are shown below: lane 1, SC pRYG DNA marker; lanes 2 and 3, 5 μg/mL; lanes 4 and 5, 0.5 μg/mL; lanes 6 and 7, 0.1 μg/mL; lanes 8 and 9, 0.05 μg/mL; lane 10, none (negative control); and lane 11, relaxed pRYG DNA marker.

grape cell culture crude extract and TP fractions can be potent chemopreventive agents via their inhibition of the catalytic activity of human DNA topoisomerase II.

The minimum concentration that inhibited 50% of the topoisomerase II catalytic activity (IC_{50}) was further determined with grape cell crude extracts and TP fractions 3−6 in the range of 0.05−5 μg/mL (TP-5 was not included in this test due to the low amount of dry mass available). All tested compounds showed dose-dependent inhibition of topoisomerase II catalytic activity. In other words, the catalytic activity of human DNA topoisomerase II that converts SC DNA to relaxed DNA was interrupted by the presence of TP-4 (0.05−1 μg/mL) and TP-6 (0.05−5 μg/mL) in a dose-dependent manner as shown in **Figures 5** and **6**, respectively. Grape cell culture crude extracts and tested TP fractions showed 18−64-fold more potent inhibition of catalytic activity of topoisomerase II in terms of IC_{50} (a range of 0.28−1.02 μg/mL) than resveratrol (IC_{50} of 18.0 μg/mL), a well-known chemopreventive agent (*2*, *8*) and topoisomerase II inhibitor (*4*, *42*) from grapes (**Figure 7**). According to Cho et al. (*4*), resveratrol was one of the potent chemopreventive agents with effective inhibition of catalytic activity on mammalian topoisomerase II (IC_{50} of 15 μg/mL) along with 1,4-phenylene bis(methylene) selenocyanate (pXSC) (0.125 μg/mL), ellagic acid (0.7 μg/mL), quercetin (6.9 μg/mL), genistein (24 μg/mL), and some others (a range of 0.25−25 μg/mL). In the present study, the most potent topoisomerase II catalytic inhibitors in a descending order of potency were TP fractions 4 and 6 (0.28−0.29 μg/mL), TP-3 (0.74 μg/mL), crude extract (1.02 μg/mL), and resveratrol (18.0 μg/mL). These results were in agreement with previously reported values for resveratrol (*4*). Interestingly, TP fractions 4 and 6 were more potent than other polyphenolic chemopreventive agents such as ellagic acid, quercetin, genistein, and resveratrol. These results suggest that there is synergism among compounds found in the

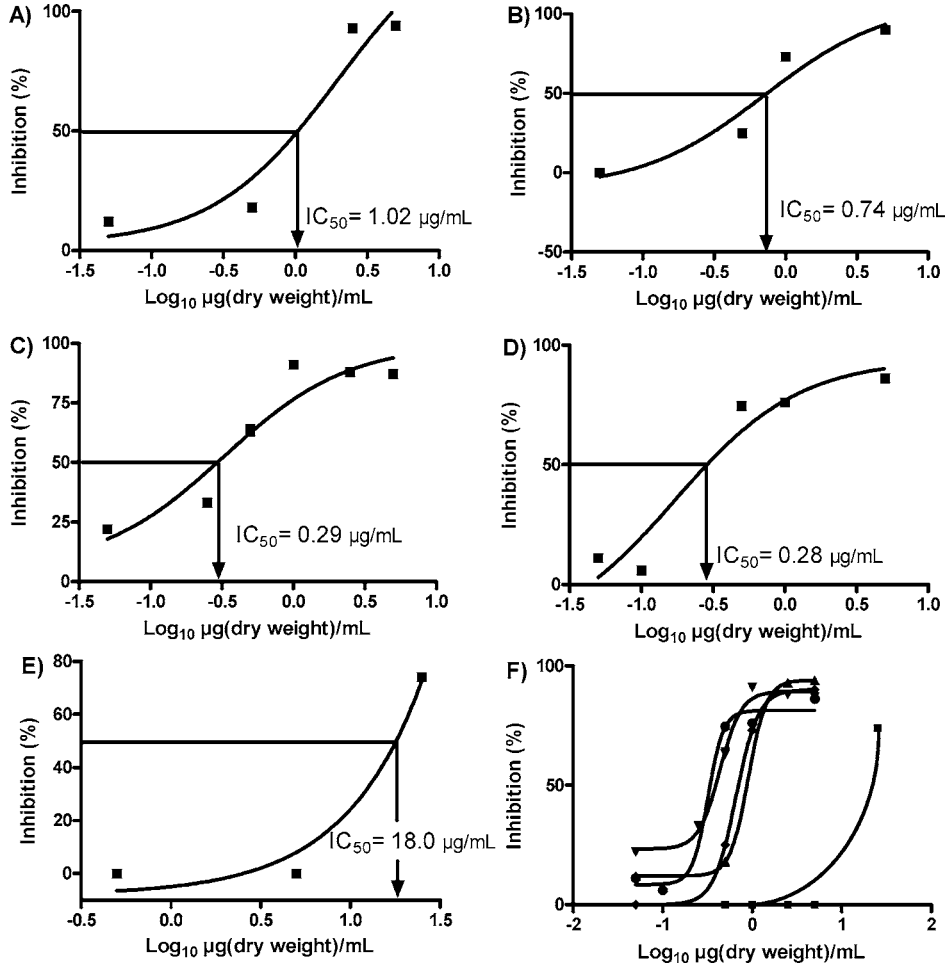

Figure 7. Catalytic inhibition (%) of grape cell crude extract and selected TP fractions on human DNA topoisomerase II. IC_{50} values were determined by the relaxation assay. (**A**) Crude extract; (**B**) TP-3; (**C**) TP-4; (**D**) TP-6; (**E**) resveratrol; and (**F**) overlaid data for crude extract (▲), TP-3 (♦), TP-4 (▼), TP-6 (●), and resveratrol (■). Each value is the average of at least duplicate measurements from two independent experiments.

extract and in TP fractions 4 and 6. In addition, there may also be other potent topoisomerase inhibitors present, which have not been previously reported. Our recent data demonstrate potent topoisomerase II catalytic inhibition by epicatechin gallate, present in TP-4. The side effects due to topoisomerase II catalytic inhibitors including genotoxicity and carcinogenicity have been reported in the case of topoisomerase II poisons rather than catalytic inhibitors (*43*), since topoisomerase II poisons lead to permanent DNA breakage and subsequent apoptosis. Chromosome breakage is also found from topoisomerase II poisons but not catalytic inhibitor treatment (*44*).

In summary, the inhibition of catalytic activity of topoisomerase II by grape cell culture crude extract and TP fractions 2−6 was significantly higher than the activity of quercetin at 5 μg/mL but in terms of IC_{50} value, both TP-4 and TP-6 were 4-fold more potent than crude extract. TP-4 and TP-6 were 62−64-fold more potent than resveratrol, respectively (**Figure 7F**). In addition, the antitopoisomerase II activity of grape crude extract and subfractions did not correlate with antioxidant capacity measured by FRAP assay (**Figure 8**). These results suggest that in the case of grape cell culture extracts, antitopoisomerase II catalytic activity is independent of antioxidant capacity.

In conclusion, both HPLC and LC-ESI/MS provided valuable tools for the characterization of bioactive fractions of grape cell cultures. Grape cell cultures accumulated simple phenolics, stilbenes, and flavonoids such as anthocyanins, procyanidins,

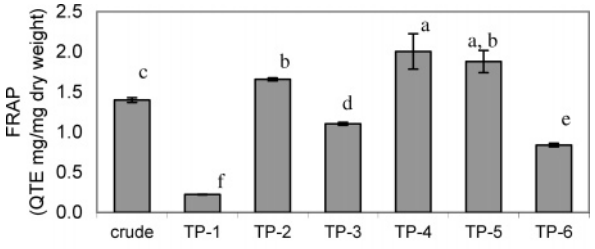

Figure 8. Antioxidant capacity of grape cell crude extract and TP fractions measured by the FRAP assay. The values represent mg of QTE per mg of dry sample. Data are means ± SEM ($n = 4$). Values with different letters (a–f) are statistically different ($p < 0.0001$).

flavanones, and flavonols. Our analysis determined the previously unreported presence of cyanidin-3,5-diglucoside, malvidin-3-acetylglucoside, peonidin-3-coumaryl-5-diglucoside, gallocatechin, epigallocatechin, procyanidin B_1(PB_1), PB_2, PB_5, procyanidin dimer digallate, PC_1, myricetin, and rutin in grape cell cultures, many of which have been reported to be antioxidant and/or chemopreventive agents.

Furthermore, we report for the first time the potent inhibition of catalytic activity of human DNA topoisomerase II by grape cell culture crude extract and subfractions. Our results suggest that these fractions are potent topoisomerase II catalytic inhibitors, via a mechanism independent of antioxidant capacity. The antitopoisomerase II catalytic activity of the most potent fractions, TP-4 and TP-6, was significantly higher than the

previously reported activity of recognized chemopreventive agents such as ellagic acid, quercetin, genistein, and resveratrol. Further studies are needed to investigate any possible interactions between bioactive constituents in both TP fractions 4 and 6, because they demonstrate much more potent antitopoisomerase II catalytic activity than any previously reported polyphenolic compounds.

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