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Catalytic Inhibition of Human DNA Topoisomerase II by Interactions of Grape Cell Culture Polyphenols

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Previously, we isolated mixed polyphenolic fractions on a toyopearl matrix (TP-2 to TP-6) from grape cell cultures that were highly potent catalytic inhibitors in a human DNA topoisomerase II assay for cancer chemoprevention. The objectives of this study were to evaluate the potency of, and potential interactions between, individual fractions and some of the purified bioactive polyphenols that comprise these fractions on human DNA topoisomerase II catalytic activity. Treatments that combined anthocyanin-rich fractions (TP-2; 0.5 or 2.0 μg of dried material/mL), fractions containing catechins, procyanidin dimers, and flavanones (TP-4; 0.25 μg of dried material/mL), and/or fractions enriched with procyanidin oligomers and polymers (TP-6; 0.15 or 0.5 μg of dried material/mL) showed additive effects toward catalytic inhibition of the enzyme. Epicatechin gallate (IC_{50} = 0.029 μM), myricetin (0.39 μM), procyanidin B_2 (PB_2, 4.5 μM), and resveratrol (65.7 μM), constituents of the most bioactive mixed fraction from grape cell culture (TP-4), each individually provided potent catalytic inhibition of topoisomerase II. In addition, potentiating interactions between the PB_2 and the other polyphenolic constituents mentioned above and between myricetin and resveratrol were clearly demonstrated. A synergistic interaction between myricetin and resveratrol was also confirmed with isobolographic analysis at a molar ratio of 1:70.

KEYWORDS: Chemopreventive agents; grape cell culture; isobolographic analysis; resveratrol; synergistic interactions; topoisomerase II catalytic inhibition; *Vitis*

INTRODUCTION

Biologically active phytochemical constituents from grapes, grape products, and grape cell cultures have demonstrated chemopreventive potential (*1*, *2*). Epidemiological studies suggest that polyphenol-rich fruits and wine may diminish cancer risk and cardiovascular disease (*3*, *4*). Resveratrol, a well-known chemopreventive agent present in grapes and wine, demonstrated inhibition of tumor growth and metastasis in hepatoma-bearing rats (*5*).

The bioactive polyphenolic compounds occur in mixtures within edible foods and are ingested in complex mixtures; interactions between the co-occurring phytochemicals may potentiate biological activity. For example, combinations of resveratrol and quercetin in grapes synergistically induced apoptosis in a human pancreatic carcinoma cell line (*6*). The effects of resveratrol and quercetin were potentiated by ethanol, which suppressed inducible nitric oxide (NO) synthase gene expression and NO production by macrophages, suggesting potential health benefits of red wine in atherosclerosis and tumor initiation (*7*). A synergistic interaction of ellagic acid and quercetin was also significant in the reduction of proliferation and viability and the induction of apoptosis in human leukemia cells (*8*). A combination of two isoflavones, genistein and daidzein, inhibited neoplastic transformation in a synergistic manner (*9*).

DNA topoisomerase II is essential for cell division and proliferation since it is required for the completion of mitosis by regulating DNA topology. Highly proliferating tumor cells were reported to express the enzyme at 25−300 times higher levels than those of quiescent cells (*10*), suggesting topoisomerase II as a good target enzyme for new anticancer drug discovery. Currently, the exact mechanism of chemoprevention by polyphenolic compounds exhibiting anti-topoisomerase activities has not been well-defined. Nonetheless, several 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 (*11*). Myricetin, daidzein, baicalein, fisetin, biochanin, and galangin have been reported to have topoisomerase II catalytic inhibitory activity (*12*, *13*). Recently, mate (*Ilex paraguariensis*) tea extract rich in polyphenols was found to act as a topoisomerase II catalytic inhibitor and also showed somewhat selective cytotoxicity on malignant oral carcinoma cells tested (*14*).

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Previously, vacuum liquid chromatography (VLC) on a toyopearl (TP) matrix yielded fractions of grape cell culture extract, which provided significant catalytic inhibition of human topoisomerase II (*15*). Partial chemical characterization of TP fractions revealed that TP-2 was an anthocyanin-rich fraction, TP-4 was comprised of polyphenols including epicatechin gallate (ECG), myricetin, procyanidin B_2 (PB_2), and resveratrol, and TP-6 contained a high concentration of oligomeric procyanidins that were coeluted as a large unresolved peak (*15*, *16*).

The objectives of this research were to evaluate the potency of, and possible interactions between, biologically active mixed fractions and individual polyphenols present in grape cell culture fractions on human DNA topoisomerase II catalytic activity.

MATERIALS AND METHODS

Grape Cell Culture Protocol. Cell suspension cultures were initiated from highly pigmented callus cultures of grape (a *Vitis* hybrid, Bailey Alicant A), which were donated by Dr. M. Shuler (Department of Chemical Engineering, Cornell University, Ithaca, NY). Cell suspensions were harvested after 2 weeks of culture under continuous fluorescent light (158 ± 2 μmol m^{-2} s^{-1}) at 25 °C as previously described (*15*). Harvested cells were held at −80 °C.

Fractionation of Grape Cell Crude Extract. Cell crude extract was prepared by extraction of frozen cells with 70% (v/v) aqueous acetone and was further fractionated using VLC on HW-40F TP resin polymer (TP) (TOSOH Bioscience LLC, Montgomeryville, PA) as previously described (*15*). Six fractions (TP-1 to TP-6) were collected.

Chemicals and Reagents. Myricetin [≥95%, high-performance liquid chromatography (HPLC) grade] and PB_2 (>91%, reagent grade) were purchased from Fluka Chemical Corp. (Milwaukee, WI) and ChromaDex Inc. (Santa Ana, CA), respectively. (−)-ECG (≥98%, HPLC grade) and resveratrol (>99%) were purchased from Sigma Chemical Co. (St. Louis, MO). A topoisomerase II drug screening kit (including etoposide) and human DNA topoisomerase II were purchased from TopoGEN, Inc. (Port Orange, FL). Certified molecular biology agarose was obtained from Bio-Rad (Hercules, CA). A 10× Tris acetate-EDTA (TAE) buffer and other chemicals needed for this assay were obtained from Sigma Chemical Co.

Human Topoisomerase II Catalytic Activity. Topoisomerase II catalytic activity was monitored via electrophoresis using a topoisomerase II drug screening kit (TopoGEN, Inc.) as previously reported (*15*). Briefly, substrate supercoiled pRYG DNA (0.25 μg) was incubated with 4 units (2 μL) of human DNA topoisomerase II in the presence of test materials (2 μL) at 37 °C for 75 min. Twenty microliters of the reaction mixture contained 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mM dithiothreitol. Electophoresis was conducted 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) and run at 66 V (2 V/cm) for 5 h. After staining the 1% agarose gel in ethidium bromide, supercoiled DNA (pRYG) was quantified by KODAK 1D Image Analysis Software version 3.5 (Eastman Kodak Co., Rochester, NY) after digital image acquisition using Kodak Image Station 440 CF (Eastman Kodak Co.). The inhibitory activity was expressed as relative activity of topoisomerase enzyme in the presence of the test materials in comparison to that in the negative control solution.

Interaction Study. Possible interactions between bioactive constituents from an anthocyanin-rich fraction (TP-2), a catechin, a procyanidin dimer and flavanone-containing fraction (TP-4), and a procyanidin-enriched fraction (TP-6) were tested. The criteria used to choose the ratio in each combination, for example, TP-2/TP-4 at a 2:1 ratio, was based on the relative weight of each fraction present naturally in the grape cell culture extract. Combinations of TP-2/TP-4 were tested on the catalytic activity of human DNA topoisomerase II at a 2:1 ratio, TP-2/TP-6 at a 4:1 ratio, and TP-4/TP-6 at a 5:3 ratio. Six binary combinations of four pure bioactive polyphenols naturally present in one of the most potent fractions (TP-4) from grape cell culture were further evaluated for their catalytic inhibition. Calculated and experimental inhibition values (%) by binary combinations of these polyphenolic components were compared to investigate potentiating interactions. Experimental effects that were significantly stronger than the calculated or additive values for binary combinations were defined as synergistic or "more than additive" interactions as previously reported (*6*, *8*).

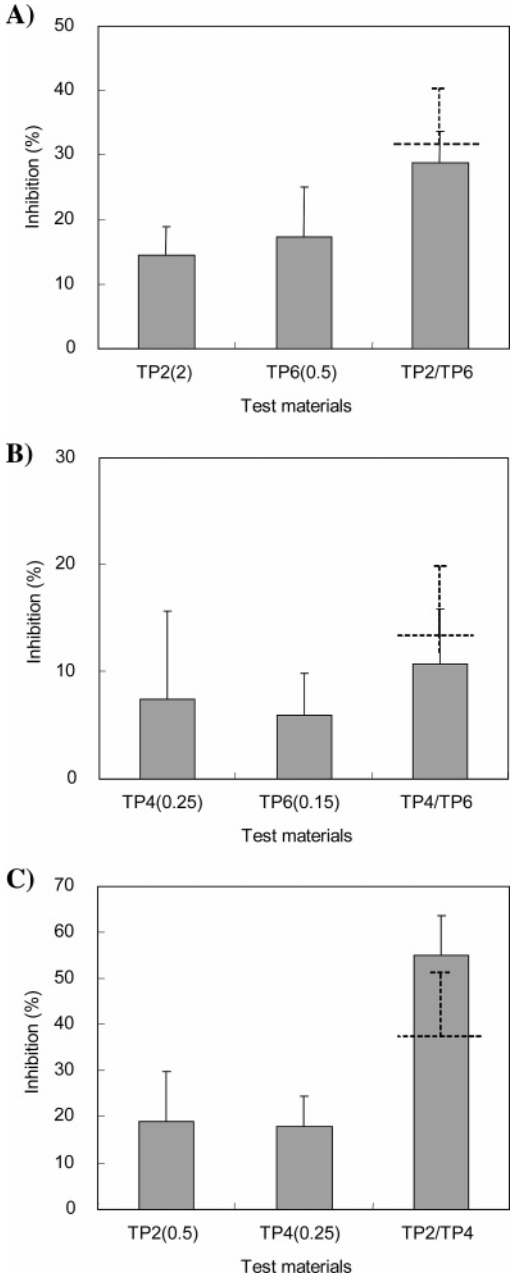

Figure 1. Catalytic inhibition (%) on human DNA topoisomerase II after treatment with (**A**) TP-2 at 2 μg of dried material/mL [TP2 (2)], TP-6 at 0.5 μg of dried material/mL [TP6 (0.5)], and the combination of TP-2 at 2 μg of dried material/mL and TP-6 at 0.5 μg of dried material/mL (TP2/TP6); (**B**) TP-4 at 0.25 μg of dried material/mL [TP4 (0.25)], TP-6 at 0.15 μg of dried material/mL [TP6 (0.15)], and the combination of TP-4 at 0.25 μg of dried material/mL and TP-6 at 0.15 μg of dried material/mL (TP4/TP6); and (**C**) TP-2 at 0.5 μg of dried material/mL [TP2 (0.5)], TP-4 at 0.25 μg of dried material/mL, and the combination of TP-2 at 0.5 μg of dried material/mL and TP-4 at 0.25 μg of dried material/mL (TP2/TP4). Data represent means ± SEM ($n \geq 2$). The added values of the combined compounds are shown as dotted lines; $p > 0.05$ (*t*-test).

Isobolographic Analysis. For the components myricetin and resveratrol, a further isobolographic analysis (*17*) was performed to confirm the synergistic interaction on topoisomerase II catalytic inhibition. Because an isobologram must be constructed in the linear range (using linear regression) and the IC_{35} value of these compounds comparing

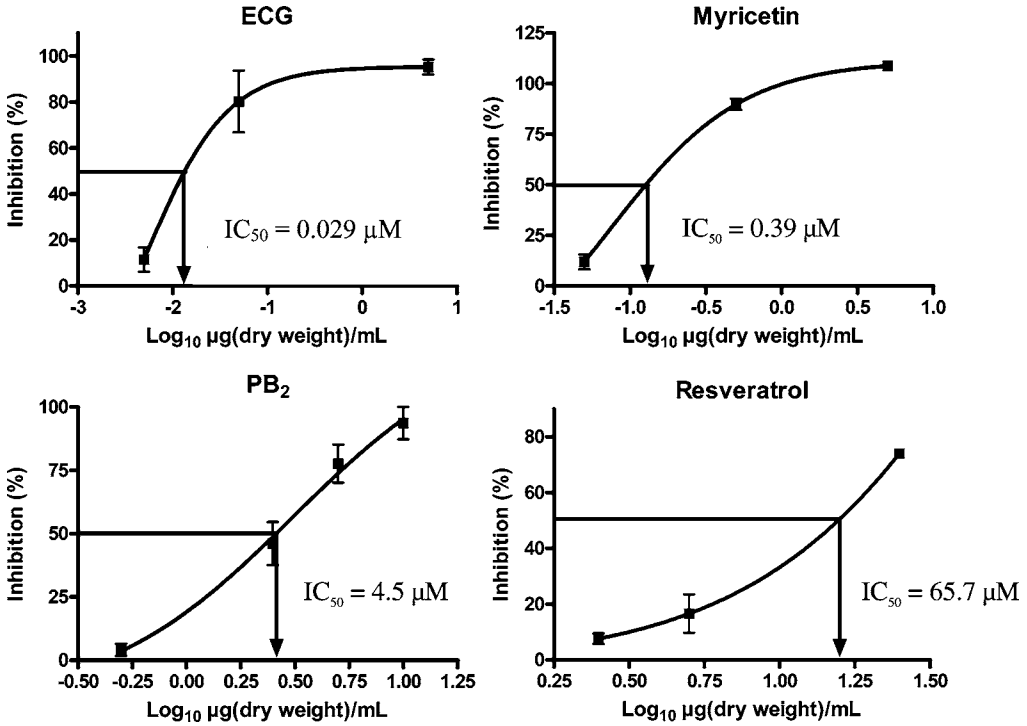

Figure 2. Catalytic inhibition (%) on human DNA topoisomerase II after treatment with pure polyphenols present in TP fraction 4 (TP-4). In descending order, the potency was ECG (IC_{50} = 0.029 μM), myricetin (IC_{50} = 0.39 μM), PB_2 (IC_{50} = 4.5 μM), and resveratrol (IC_{50} = 65.7 μM). IC_{50} values were determined by the relaxation assay as previously described (*15*). Each value represents means ± SEM ($n \geq 4$) from two independent experiments.

concentration and inhibition was in the middle of the linear range, IC_{35} was selected as the standard for construction of the isobologram. In an isobologram, a straight line connecting the IC_{35} values for two compounds will predict the IC_{35} value of an additive effect in a combination at various concentrations in the same ratio. If the actual, experimentally determined IC_{35} deviates significantly to the left of this predictive additive line, then the potentiating interaction is confirmed as a synergy (*8*, *17*). In this study, the IC_{35} isobologram was generated by combining myricetin and resveratrol at a molar ratio of 1:70, based on estimation of the relative amount of each compound in TP-4. All IC_{35} values of tested materials were calculated by linear regression using the Prism 3.0 (GraphPad Software, San Diego, CA).

Statistical Analysis. An independent sample *t*-test was used to analyze data using SAS version 9.1 (SAS Institute Inc., Cary, NC). Differences were considered significant at $p < 0.05$. All other calculations were performed on the Prism 3.0 statistical program.

RESULTS AND DISCUSSION

Additive effects, without evidence of further potentiating interactions, were observed when combinations of TP-2 (2 μg of dried material/mL)/TP-6 (0.5 μg of dried material/mL) (**Figure 1A**) and TP-4 (0.25 μg of dried material/mL)/TP-6 (0.15 μg of dried material/mL) (**Figure 1B**) from grape cell culture were tested for catalytic inhibition of human DNA topoisomerase II. The resulting catalytic inhibition of enzyme after treatment with a combination of TP-2 (an anthocyanin-enriched fraction) and TP-4 (a catechin, procyanidin dimer, and flavanone-containing fraction) suggested a tendency toward a potentiating interaction; however, this interaction was not statistically significant ($p > 0.05$); thus, it was also classified as a simple additive effect in the topoisomerase II catalytic inhibition assay (**Figure 1C**). Probable synergistic antiproliferative effects from mixtures of anthocyanins, proanthocyanidins, and flavonol glycosides have been cited previously (*18*); however, the mechanisms behind these interactions may not directly relate to topoisomerase II catalytic inhibition.

Also using human DNA topoisomerase II catalytic activity, the IC_{50} values were determined for ECG (IC_{50} = 29 nM), myricetin (IC_{50} = 0.39 μM), PB_2 (IC_{50} = 4.5 μM), and resveratrol (IC_{50} = 65.7 μM), four topoisomerase II catalytic inhibitors present in one of the most potent subfractions (TP-4, IC_{50} = 0.29 μg/mL) from grape cell culture (**Figure 2**). Each polyphenolic compound inhibited the activity of topoisomerase II in a dose-dependent manner.

This is the first report of IC_{50} values of ECG and PB_2 on human DNA topoisomerase II catalytic activity. Moreover, ECG was found to be a potent catalytic inhibitor of topoisomerase II at nanomolar concentrations. The potency of ECG and myricetin suggests that topoisomerase II catalytic inhibition as a contributing mechanism for the potent chemopreventive activity of these two phytochemicals (*19*, *20*).

When interactions between phytochemicals were tested, PB_2 showed synergistic or "more than additive" interactions (*8*) with other polyphenols such as ECG (**Figure 3A**), myricetin (**Figure 3B**), and resveratrol (**Figure 3C**). Previously, the protective synergistic interaction of resveratrol and catechin from β-amyloid peptide-induced cytotoxicity on rat pheochromocytoma (PC12) cells was reported (*21*). Furthermore, myricetin and resveratrol interacted synergistically to inhibit topoisomerase II catalytic activity (**Figure 4**), which was also confirmed by an isobolographic analysis at a molar ratio of 1:70 (**Figure 5**). The synergistic interaction of quercetin and resveratrol was reported in reducing proliferation of an oral squamous carcinoma cell line (*22*) and inducing apoptosis in a human pancreatic carcinoma cell line (*6*). These polyphenols also synergistically interacted in the induction of apoptosis via the activation of caspase-3 in a human leukemia cell line (*8*). Because myricetin present in TP-4 has a similar structure to quercetin (both members of the flavonol subclass), the suspected synergistic interaction between myricetin and resveratrol in the topoisomerase II catalytic inhibition assay was selected for further

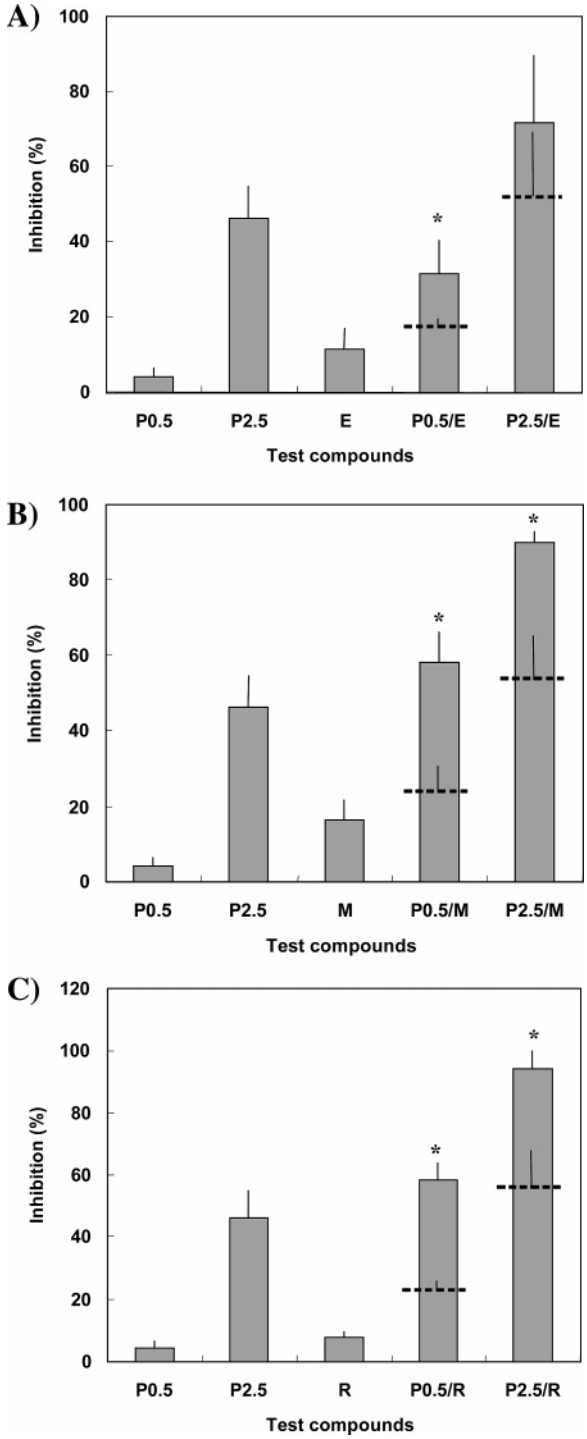

Figure 3. Catalytic inhibition (%) on human DNA topoisomerase II after treatment with (**A**) PB_2 at 0.5 (0.86 μM, P0.5) or 2.5 μg/mL (4.3 μM, P2.5), ECG at 0.005 μg/mL (11.3 nM, E), or the combination of P at 0.5 μg/mL and E at 0.005 μg/mL (P0.5/E) or P at 2.5 μg/mL and E at 0.005 μg/mL (P2.5/E); (**B**) PB_2 at 0.5 (0.86 μM, P0.5) or 2.5 μg/mL (4.3 μM, P2.5), myricetin at 0.05 μg/mL (0.16 μM, M), or the combination of P at 0.5 μg/mL and M at 0.05 μg/mL (P0.5/M) or P at 2.5 μg/mL and M at 0.05 μg/mL (P2.5/M); and (**C**) PB_2 at 0.5 (0.86 μM, P0.5) or 2.5 μg/mL (4.3 μM, P2.5), resveratrol at 2.5 μg/mL (11.0 μM, R), or the combination of P at 0.5 μg/mL and R at 2.5 μg/mL (P0.5/R) or P at 2.5 μg/mL and R at 2.5 μg/mL (P2.5/R). Data represent means ± SEM ($n \geq 8$). The added values of the combined compounds are shown as dotted lines; *$p < 0.05$ (*t*-test).

confirmation with an isobolographic analysis. In brief, the straight, solid line drawn between IC_{35} points with myricetin

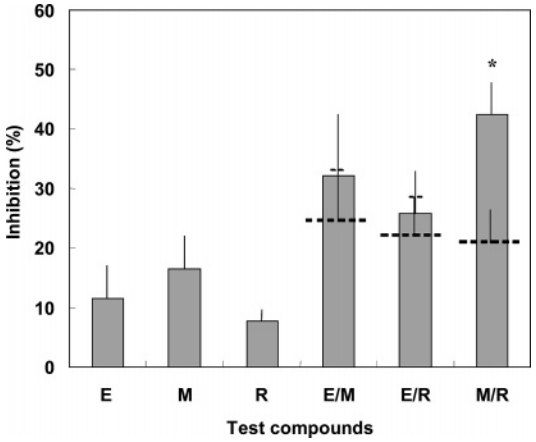

Figure 4. Catalytic inhibition (%) on human DNA topoisomerase II after treatment with ECG at 0.005 μg/mL (11.3 nM, E), myricetin at 0.05 μg/mL (0.16 μM, M), resveratrol at 2.5 μg/mL (11.0 μM, R), the combination of E at 0.005 μg/mL and M at 0.05 μg/mL (E/M), E at 0.005 μg/mL and R at 2.5 μg/mL (E/R), or M at 0.05 μg/mL and R at 2.5 μg/mL (M/R). Data represent means ± SEM ($n \geq 8$). The added values of the combined compounds are shown as dotted lines; *$p < 0.05$ (*t*-test).

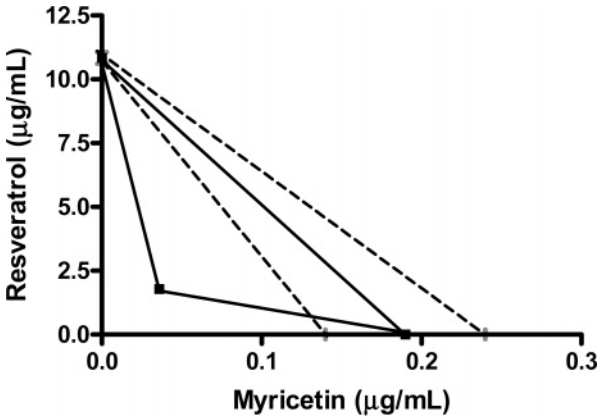

Figure 5. IC_{35} isobologram for the synergistic interaction of myricetin and resveratrol (at a molar ratio of 1:70) with respect to its topoisomerase II catalytic inhibition. The line of additivity is shown with its 95% confidence limits.

alone and resveratrol alone predicted the IC_{35} values of all combinations in an additive effect. The empirical IC_{35} from these combinations deviated toward the lower left from the additive line, confirming the synergistic interaction between these compounds (**Figure 5**).

This is the first report of the synergistic interaction of grape polyphenols on catalytic inhibition of human DNA topoisomerase II. Synergistic or more than additive interactions between PB_2 and the other polyphenols on any potential chemopreventive activities have not been previously reported. A synergistic interaction between myricetin and resveratrol on potential chemoprevention was confirmed by isobolographic analysis for the first time using the topoisomerase II inhibition assay. Highly active catalytic inhibition of TP-4 on topoisomerase II may be due to synergistic interactions between polyphenols such as ECG, myricetin, PB_2, and resveratrol. Combining bioactive polyphenolic components of TP-4 indicated synergistic interactions on topoisomerase II catalytic inhibition, which suggests that the polyphenolic components from grape cell culture fraction have potential as chemopreventive agents.

It is well-known that topoisomerase II is a target for many anticancer drugs through several mechanisms such as the

stabilization and accumulation of covalent complexes between topoisomerase II and DNA or by inhibiting the catalytic activity of the enzyme and interfering with the enzyme turnover; all of these mechanisms will result in cell death. The compounds that inhibit the catalytic activity of topoisomerase II but do not stabilize the formation of topoisomerase II·DNA complexes are clinically useful in the treatment of cancer (*23*). Snyder and Gillies (*12*) have postulated that the genotoxicity of most flavonoids arises via DNA intercalation and topoisomerase II inhibition, likely mediated through metabolism to flavonoid quinones. Some polyphenols may also act by inhibiting the enzymatic activity of topoisomerase II and causing cell death. More studies are needed to better understand the biological and chemical mechanisms of flavonoids and topoisomerase II inhibition. Moreover, given the wide clinical use of topoisomerase II-targeted agents, mechanistic issues related to polyphenol action clearly merit further investigation (*12*).

In conclusion, this study clearly demonstrated the potentiating interactions between PB_2 and other polyphenols such as ECG, myricetin, and resveratrol, as well as between myricetin and resveratrol. A synergistic interaction between myricetin and resveratrol was also confirmed with isobolographic analysis. This suggests that synergistic interactions of polyphenols in the mixture (TP-4) on human DNA topoisomerase II catalytic inhibition may contribute to its potential as a possible chemopreventive agent.

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