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Catalytic Inhibition of Eukaryotic Topoisomerases I and II by Flavonol Glycosides Extracted from *Vicia faba* and *Lotus edulis*

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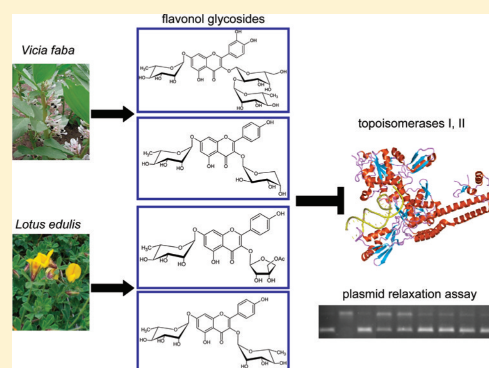
ABSTRACT: Topoisomerases are essential enzymes involved in all processes of DNA metabolism, and their inhibitors have been identified as potential anticancer agents. The present study examined the effect of nine polyphenolic compounds derived from parts of two unique varieties of the Leguminosae, *Vicia faba* and *Lotus edulis*, on the activity of eukaryotic topoisomerases. We identified polyphenolic compounds that act as catalytic inhibitors of wheat germ topoisomerase I (IC_{50}: 120−350 μM), human topoisomerase I (IC_{50}: 110−260 μM), and human topoisomerase II (IC_{50}: 240−600 μM) activities. Some compounds inhibited all enzymatic activities to a similar extent, while others exhibited specificity toward individual enzymes. The strongest catalytic inhibitor of all the examined enzymes was a kaempherol glycoside with an acetyl group linked to a sugar moiety. In addition, this compound inhibited the growth of human cancer cell lines MCF7, HeLa, and HepG2. The inhibition of topoisomerase I and II activities observed by the specific compounds possibly implies a role as potential agents in the prevention and therapy of cancer.



Legumes constitute an important component of many diets worldwide because of their high nutritive value and their beneficial effects toward various diseases, including diabetes, cardiovascular disease, and certain types of cancer as exhibited by in vitro and epidemiological studies.[1−7] Legumes are a rich source of phenolic compounds such as flavonoids, phenolic acids, and lignans.[8−13] These phytochemical compounds exhibit antioxidant activities and have been proposed to exert chemopreventive actions through various mechanisms.[14−18]

Topoisomerases I and II are key enzymes involved in the processes of DNA replication, transcription, recombination, and maintenance of genome stability and play a vital role during cell proliferation and differentiation.[19−21] Their catalytic cycle includes DNA strand breakage (one strand by topoisomerase I or both strands by topoisomerase II), formation of a covalent complex with DNA, followed by DNA strand passage, and the final religation step of the initial DNA strand.[21] DNA topoisomerases are the molecular targets of many antimicrobial and anticancer agents.[22−26] Inhibitors of eukaryotic topoisomerases are divided into two classes, depending on their mechanism of inhibition: (i) catalytic inhibitors, or topoisomerase antagonists, which prevent formation of enzyme−DNA complex and consequently enzymatic turnover, and (ii) poisons, or topoisomerase cytotoxic agents, which stabilize the enzyme−DNA complex and prevent the religation step of the reaction. Commonly topoisomerase I and II poisons are used as chemotherapeutic agents;[22−24] however topoisomerase catalytic inhibitors that exhibit antitumor effects have been identified in cell culture and animal studies.[27−32]

We have previously reported the isolation of polyphenolic fractions and further identified pure polyphenolic compounds derived from two unique varieties of the Leguminosae, *Vicia faba* and *Lotus edulis*.[33] These polyphenolic fractions exhibited significant antioxidant properties and were protective against DNA damage induced by reactive oxygen species. In addition, some of these polyphenolic fractions inhibited the catalytic activity of wheat germ topoisomerase I. In extending this study, pure polyphenolic compounds were evaluated for their capacity to inhibit all eukaryotic DNA topoisomerases, including wheat germ topoisomerase I, human topoisomerase I, and human topoisomerase II. With a view to identify potential cancer chemopreventive agents we report herein flavonoid compounds that inhibit catalytically all examined topoisomerases.

RESULTS AND DISCUSSION

Polyphenolic Compounds Isolated from *V. faba* and *L. edulis*. In the context of identifying the biological properties of phytochemical compounds, with interest in chemopreventive properties, the present study examined the effect of pure polyphenolic compounds isolated from *V. faba* and *L. edulis* on the activity of eukaryotic topoisomerases, including wheat germ topo I, human topo I, and human topo II. The structures of polyphenolic compounds identified in methanolic fractions of *V. faba* and *L. edulis* are presented in Figure 1. The detailed isolation and identification of these compounds have been

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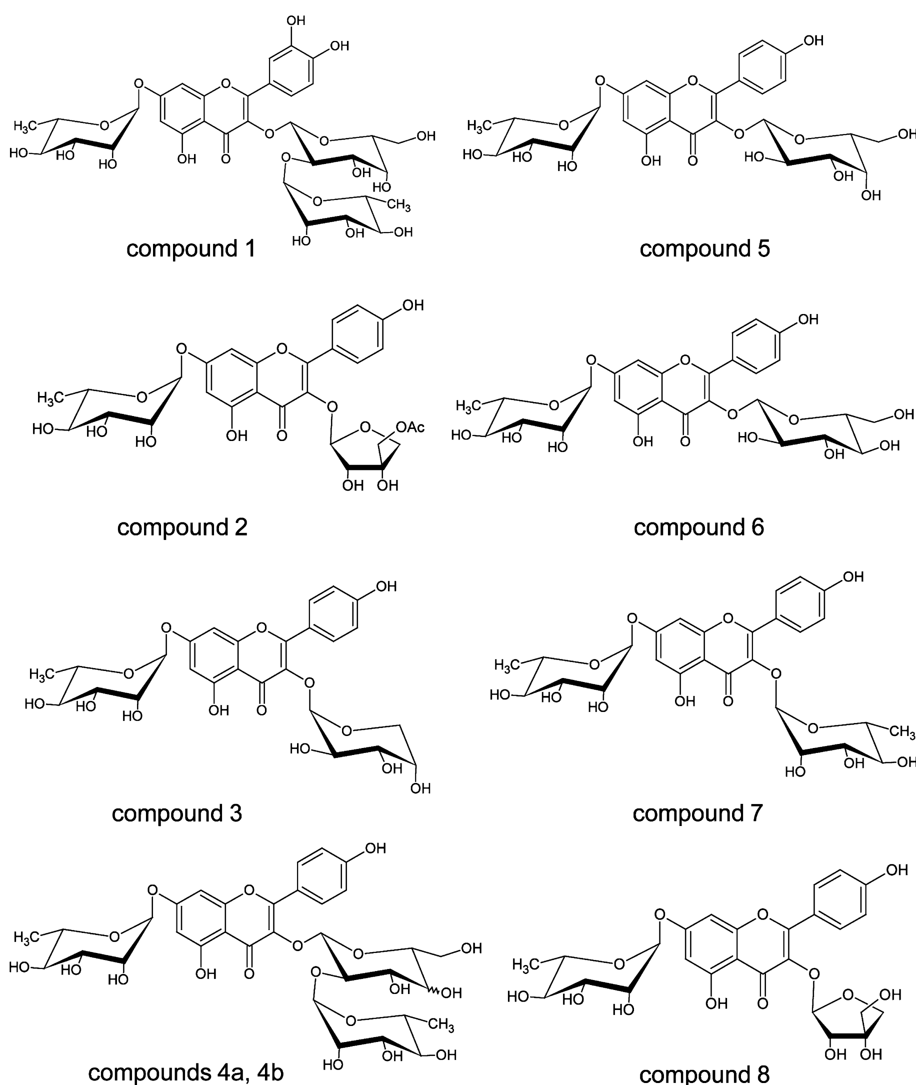

Figure 1. Polyphenolic compounds from *V. faba* and *L. edulis* methanolic plant extracts used in this study: **1**, quercetin 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl]-7-*O*-α-L-rhamnopyranoside; **2**, kaempferol 3-*O*-(5-*O*-acetyl-α-D-apiofuranosyl)-7-*O*-α-L-rhamnopyranoside; **3**, kaempferol 3-*O*-α-L-arabinopyranosyl-7-*O*-α-L-rhamnopyranoside; **4a**,**b**, kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl]-7-*O*-α-L-rhamnopyranoside and kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl]-7-*O*-α-L-rhamnopyranoside; **5**, kaempferol 3-*O*-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside; **6**, kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside; **7**, kaempferol 3-*O*-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside; **8**, kaempferol 3-*O*-β-D-apiofuranosyl-7-*O*-α-L-rhamnopyranoside.

previously described.[33−42] All compounds are flavonoids and specifically are glycosylated forms of the flavonols quercetin and kaempferol. So far more than 200 quercetin and kaempferol glycosides have been identified in various plant sources.[43]

Inhibition of Topoisomerase I Activity by Polyphenolic Compounds. In order to identify catalytic inhibitors of topoisomerase I activity, all compounds were initially examined at the concentration of 500 μM. Five compounds (**2**, **3**, **4b**, **5**, **6**) inhibited wheat germ topo I activity, and six compounds (**2**, **3**, **4a**, **4b**, **5**, **6**) inhibited human topo I activity. Subsequently, these compounds were examined at concentrations ranging from 75 to 1000 μM for wheat germ topo I and from 100 to 500 μM for human topo I to calculate the IC_{50} values. It should be mentioned that none of the compounds affected plasmid conformation when examined alone at the highest concentration. For all assays, methanolic red grape extract (Mandilaria variety) was used as a marker of a very potent topo I inhibitor, as it exhibited 95% inhibitory activity at a concentration of 800 μg/mL.[44]

Each polyphenolic compound inhibited the activity of wheat germ topo I in a dose-dependent manner. Compound **2** was the strongest catalytic inhibitor of wheat germ topo I activity, with an IC_{50} value of 120 μM (Figure 2A). Specifically, compound **2** exhibited 44% and 99% inhibition of wheat germ topo I activity at concentrations of 75 and 1000 μM, respectively. In addition, compound **4b** inhibited wheat germ topo I activity with an IC_{50} value of 150 μM (Figure 2B). Similar catalytic inhibition of wheat germ topo I activity was recorded for compounds **5** (Figure 2C) and **3** (Figure 2D), as demonstrated by the respective IC_{50} values of 200 and 240 μM. Finally, compound **6** was the weakest inhibitor of wheat germ topo I activity (IC_{50} = 350 μM) (Figure 2E).

The strongest catalytic inhibitor of human topo I activity was compound **6**, as demonstrated by the IC_{50} value of 110 μM. Specifically, compound **6** inhibited human topo I activity at 45% and 94% when used at concentrations of 100 and 500 μM,

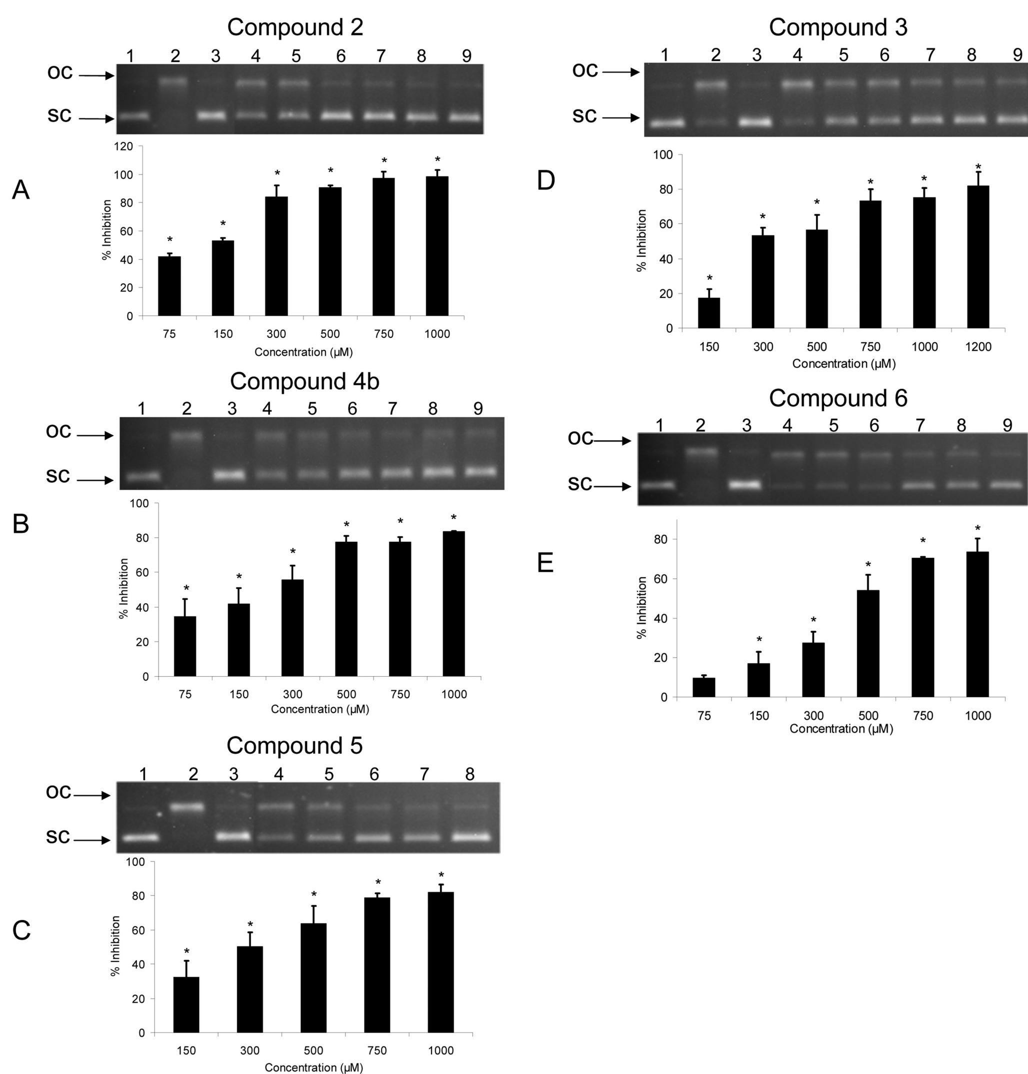

Figure 2. Inhibition of wheat germ topo I catalytic activity by polyphenolic compounds derived from *V. faba* and *L. edulis* plant extracts: (A) compound **2**; (B) compound **4b**; (C) compound **5**; (D) compound **3**; (E) compound **6**. Bluescript SK+ plasmid DNA was relaxed by 1 unit of wheat germ topo I alone (lane 2) or in the presence of 800 μg/mL methanolic red grape extract (lane 3), or 75, 150, 300, 500, 750, and 1000 μM compound, respectively (lanes 4−9) (A, B, D, E) or with 150, 300, 500, 750, and 1000 μM compound (lanes 4−8) (C). Lane 1 represents Bluescript SK+ plasmid DNA without any treatment. OC: open circular; SC: supercoiled. In each panel the percentage inhibition of wheat germ topo I activity in the presence of the indicated compound concentrations is given. The values represent means ± SEM. The percentage inhibition was calculated as described in the Experimental Section.

respectively (Figure 3A). In accordance with the strong inhibition of wheat germ topo I activity, compound **2** was a strong catalytic inhibitor of human topo I activity, with an IC_{50} value of 150 μM (Figure 3B). Inhibition of human topo I was also observed for compound **5** (IC_{50} = 180 μM) (Figure 3C). It is interesting that compound **4a** inhibited human topo I activity with an IC_{50} value of 220 μM, although it did not inhibit wheat germ topo I activity (Figure 3D). Finally, similar catalytic inhibition of human topo I activity was observed for compounds **3** and **4b**, with respective IC_{50} values of 250 and 260 μM (Figure 3E,F).

Some compounds had similar capacities in inhibiting both wheat germ and human topo I activities. This applies for compound **2**, which was the strongest inhibitor of both enzymes and also for compounds **3** and **5**. However, other compounds exhibited differences in their inhibitory abilities depending on the topoisomerase used. This was the case for compound **6**, which exhibited strong catalytic inhibition of human topo I but the weakest catalytic inhibition of wheat germ topo I. Similarly, compound **4b** was a strong inhibitor of wheat germ topo I activity but the weakest inhibitor of human topo I activity. Interestingly, compound **4a**, an epimer of compound **4b**, inhibited only human but not wheat germ topo I activity. Both wheat germ and human topoisomerase I are of eukaryotic origin; however enzymatic structural differences in combination with the different compound structures might account for the observed inhibition differences. Specifically, the sugar unit attached at the 3-OH of the kaempferol moiety appears to be responsible for the observed variation in the inhibition of topo I (human and wheat germ) activities. The presence of an apiofuranosyl moiety containing an acetyl group (compound **2**) resulted in the compound with the strongest inhibition, whereas compound **8** did not have an inhibitory effect. Glycosides containing arabinosyl (compound **3**), glucosyl (compound **6**), and galactosyl (compound **5**) moieties exhibited an inhibitory effect, while glycosides with a rhamnosyl

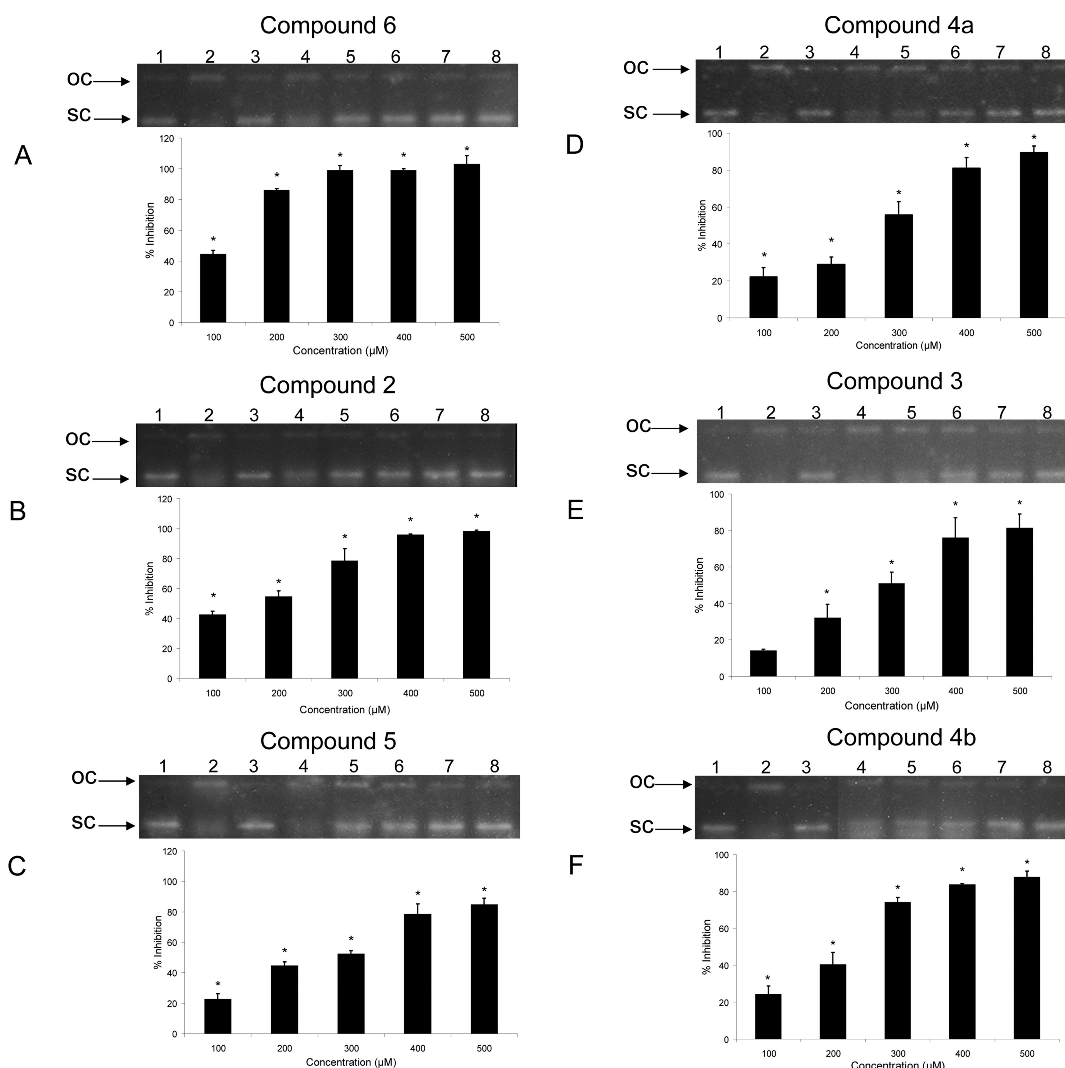

Figure 3. Inhibition of human topo I catalytic activity by polyphenolic compounds derived from *V. faba* and *L. edulis* plant extracts: (A) compound **6**; (B) compound **2**; (C) compound **5**; (D) compound **4a**; (E) compound **3**; (F) compound **4b**. Bluescript SK+ plasmid DNA was relaxed by 1 unit of human topo I alone (lane 2) or in the presence of 800 μg/mL methanolic red grape extract (lane 3), or with 100, 200, 300, 400, and 500 μM of compound, respectively (lanes 4–8) (A–F). Lane 1 represents Bluescript SK+ plasmid DNA without any treatment. OC: open circular; SC: supercoiled. In each panel the percentage inhibition of human topo I activity in the presence of the indicated compound concentrations is given. The values represent means ± SEM. The percentage inhibition was calculated as described in the Experimental Section.

moiety (compound **7**) did not. However, when rhamnose was linked to another sugar, such as galactose (compound **4b**) or glucose (compound **4a**), these disaccharide-containing kaempferol glycosides inhibited topo I activity.

Inhibition of Human Topoisomerase II Activity by Polyphenolic Compounds. The inhibition of topo II catalytic activity has been suggested to be an important parameter for the selection of cancer chemopreventive agents. To this end, some flavonoids are considered chemopreventive agents because they are able to inhibit the catalytic activity of topo II.[45] Therefore, all polyphenolic compounds were initially examined at the concentration of 500 μM for their ability to inhibit human topo II activity. Inhibition was observed for four compounds (**2**, **3**, **6**, **8**), which were further examined at concentrations ranging from 100 to 1000 μM. Each compound inhibited the activity of human topo II in a dose-dependent manner. Compound **2** was the strongest inhibitor of human topo II, similarly to its effect on wheat germ and human topo I activities. Compound **2** exhibited 22% and 88% inhibition of human topo II activity at concentrations of 100 and 500 μM, respectively, yielding an IC_{50} value of 240 μM (Figure 4A). Compounds **3** and **6** inhibited human topo II with higher IC_{50} values compared to the respective values for topo I inhibition. Specifically, compound **3** exhibited an IC_{50} value of 330 μM (Figure 4B), and compound **6** was the weakest inhibitor of human topo II activity, as indicated by the IC_{50} value of 600 μM (Figure 4D). Finally, compound **8**, which had no effect on topo I (wheat germ and human) activities, inhibited human topo II activity. The IC_{50} value for compound **8** was 550 μM, based on the inhibition values of 44% and 80% at concentrations of 400 and 800 μM, respectively (Figure 4C).

The different compound efficiencies in inhibiting individual topoisomerase catalytic activities are reflected by the relative IC_{50} values (Figure 5). In previous studies flavonoids, such as quercetin and kaempferol and their glycosides, have been characterized as strong inhibitors of topo I and topo II.[46−50] This study examined pure polyphenolic compounds; therefore, it is possible to correlate structural characteristics with the

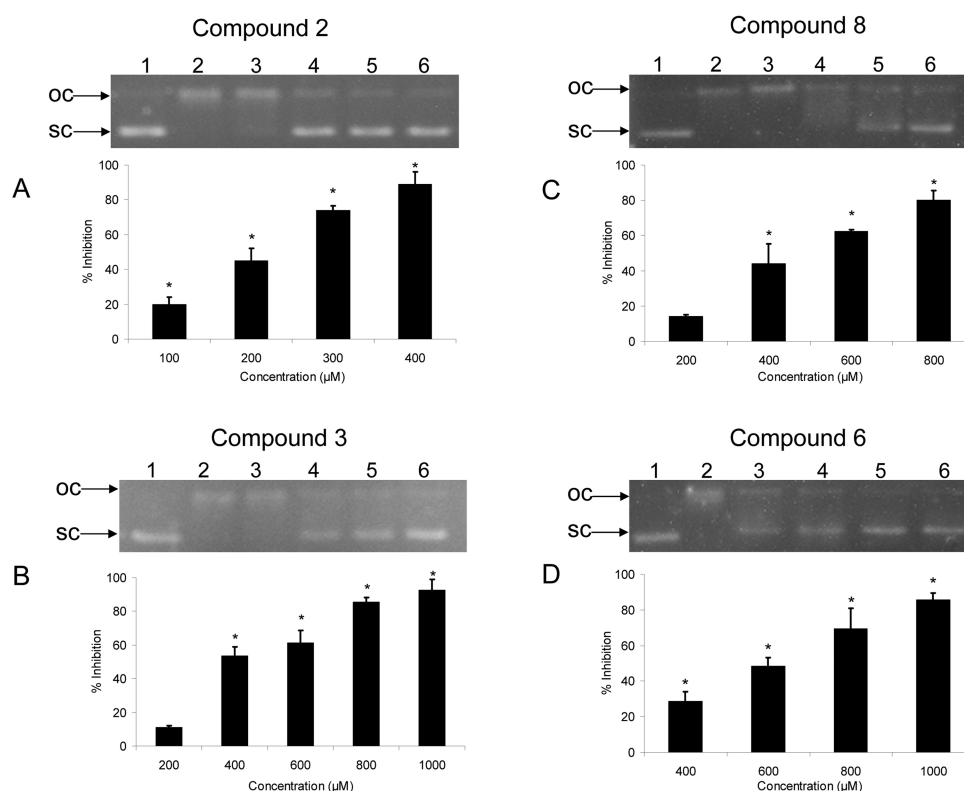

Figure 4. Inhibition of human topo II catalytic activity by polyphenolic compounds derived from *V. faba* and *L. edulis* plant extracts: (A) compound **2**; (B) compound **3**; (C) compound **8**; (D) compound **6**. Bluescript SK+ plasmid DNA was relaxed by 1.6 units of human topo II alone (lane 2) or with 100, 200, 300, and 400 μM of compound (lanes 3−6) (A) or with 200, 400, 600, and 800 μM of compound (lanes 3−6) (B, C) or with 400, 600, 800, and 1000 μM of compound, respectively (lanes 3−6) (D). Lane 1 represents Bluescript SK+ plasmid DNA without any treatment. OC: open circular; SC: supercoiled. In each panel the percentage inhibition of human topo II activity in the presence of the indicated compound concentrations is given. The values represent means ± SEM. The percentage inhibition was calculated as described in the Experimental Section.

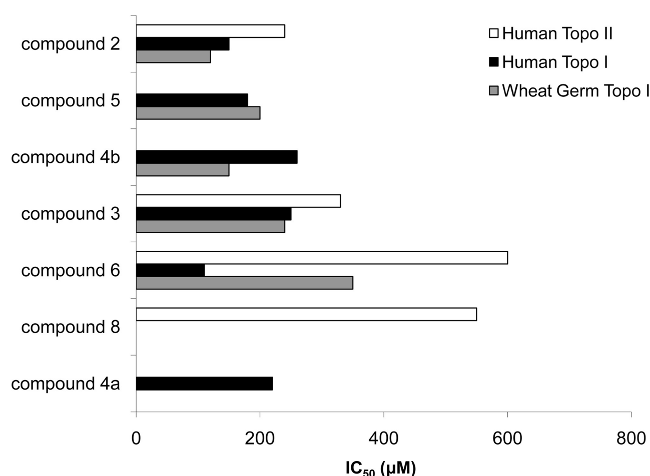

Figure 5. Inhibition of wheat germ topo I, human topo I, and human topo II catalytic activities by polyphenolic compounds derived from *V. faba* and *L. edulis* plant extracts. The average IC_{50} values (calculated from at least three independent experiments) are indicated for each compound. Note that no IC_{50} values are shown for those compounds for which no individual topoisomerase inhibition was recorded.

ability to inhibit topo I and topo II activities. The observed differences in the inhibitory effect of these compounds might be attributed to the different stereochemical conformation, the number and the availability of hydroxy groups, the positions of hydroxy glycosylations, and the different type and/or the number of sugar moieties. It has been shown that flavonoids demonstrating potent topo I and topo II inhibition require hydroxy groups at C-3, C-7, C-3′, and C-4′, a carbonyl group at C-4, and a C-2/C-3 double bond.[46] All the identified compounds that inhibit topo I and topo II have the aforementioned characteristics. As was the case for topo I inhibition, the sugar unit linked at the 3-OH of the kaempferol glycoside appears to be significant for the observed variation in human topo II inhibition. The presence of an apiofuranosyl moiety containing an acetyl group (compound **2**) resulted in the compound with the strongest topo II inhibition, whereas the apiofuranosyl moiety on its own (compound **8**) had a reduced inhibitory effect. Among the other glycosides tested only arabinosyl- (compound **3**) and glucosyl-containing (compound **6**) compounds inhibited human topo II activity, while galactosyl- and rhamnosyl (alone or as part of a disaccharide)-containing glycosides did not. The results of this study demonstrate that the structural characteristics of compound **2**, specifically the presence of the acetyl moiety, favor the interaction and result in catalytic inhibition of both topo I and topo II activities.

Numerous studies have shown that some flavonoids can inhibit and/or poison topo I and/or topo II, and most of these experiments have been conducted in vitro with purified DNA and enzymes. However, some of these studies have shown contradictory results. For instance quercetin, kampeferol, fisetin, and apigenin have been reported to act as topo I and topo II poisons.[47,51−54] Additionally, it has been demonstrated that the potency of quercetin in stabilizing the covalent topo I−DNA intermediate requires hydroxy groups at C-5 and C-7 of

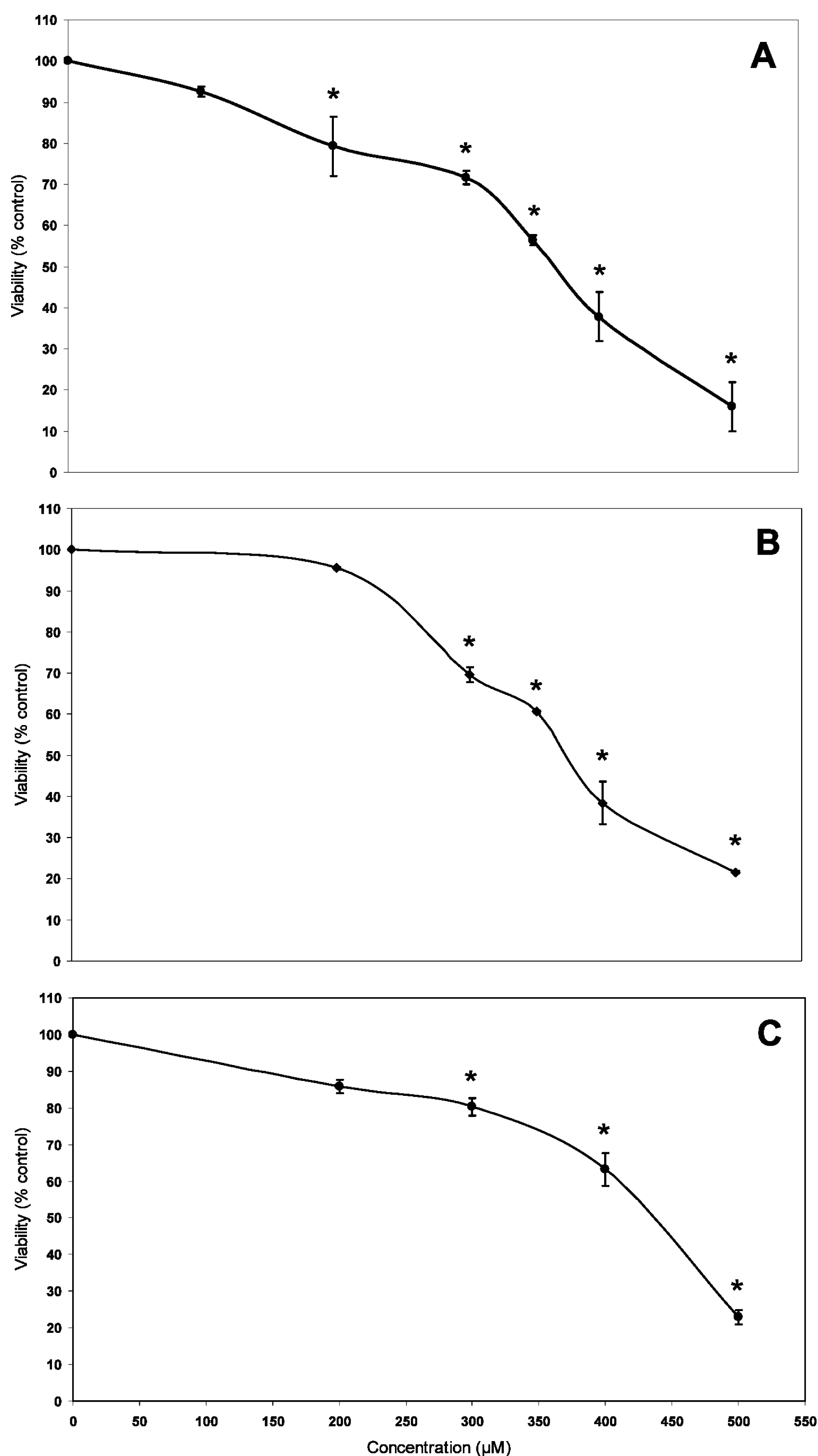

Figure 6. Effect of compound **2** on viability of (A) HeLa, (B) MCF7, and (C) HepG2 tumor cell lines. Viability was assayed as described in the Experimental Section. The values represent means ± SEM.

the phenolic A-ring.[51] However, other studies have reported that quercetin does not act as a topo I poison, but instead acts as a topo I and topo II catalytic inhibitor.[45,46,55] In this study compound **1**, which is a quercetin glycoside, did not inhibit topo I and topo II activities. It is possible that the presence of sugar moieties linked to C-3 might affect the enzymatic inhibition; alternatively compound **1** might act as a topoisomerase poison, which was not assayed by the protocol used here.

Evaluation of Cytotoxic Activity on Human Tumor Cell Lines. Compound **2** was the strongest catalytic inhibitor of all examined topoisomerases. Therefore the cytotoxicity of compound **2** was tested against the following human cell lines: MCF7 (epithelial breast adenocarcinoma), HeLa (epithelial cervix adenocarcinoma), and HepG2 (hepatocellular liver

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carcinoma). Compound **2** exhibited inhibition of cell growth of all the tested cell lines (Figure 6). The IC_{50} values were 350 μM for HeLa (Figure 6A) and MCF7 cells (Figure 6B) and 410 μM for HepG2 cells (Figure 6C). For each experiment cell cultures containing 100 μM etoposide were used as a positive control, as they exhibited 100% inhibition of cellular growth (data not shown). Etoposide is a known inhibitor of topoisomerase II and a commonly used anticancer drug. It is important to note that the IC_{50} values estimated by the inhibition of cell growth (350–410 μM) are higher compared to the IC_{50} values estimated by the plasmid relaxation assay (120–240 μM). This observation is not surprising, because a cellular system is less easily accessible compared to the in vitro plasmid system. Similarly to compound **2**, previous studies have described the cytotoxic activity of kaempferol glycosides derived from various plants against cancer cell lines.[56−59]

It is important to note that compound **2** exhibits strong antioxidant capacities. A previous study demonstrated that compound **2** has significant free radical (DPPH$^\bullet$) scavenging capacity and exerts protective activity against free radical-induced DNA damage (DNA strand breaks induced by hydroxy and peroxy radicals).[33] On the basis of the above findings, it would be important to further identify the structural domains of compound **2** involved in its various abilities, for instance in the interaction with eukaryotic topoisomerases or its antioxidant properties.

In conclusion, this study reports the catalytic inhibition of eukaryotic and human topo I and human topo II activities from polyphenolic compounds identified in two unique varieties of the Leguminosae. The ability of these polyphenolic compounds to inhibit the activity of all examined topoisomerases, in combination with the cytotoxic activity and with their previously reported antioxidant properties, makes them important chemopreventive agents. Flavonoids have shown cytotoxic effects in vitro and in animal models of carcinogenesis, and some have entered clinical trials for the prevention or treatment for specific cancers.[16,60−63] Future research should focus on bioavailability studies using in vivo animal models.

■ EXPERIMENTAL SECTION

Reagents. Topoisomerase I from wheat germ was obtained from Promega (Madison, WI, USA). Recombinant human topoisomerase I (wild type) and purified human DNA topoisomerase IIα (p170 form) were purchased from Topogen (Florida, USA). All dilution and reaction buffers for human topoisomerases were purchased from Topogen (Florida, USA). All other chemicals and solvents were of the highest quality commercially available. Bluescript SK+ plasmid DNA was isolated from a large-scale bacterial culture, using the alkali lysis method.

Plant Material. The edible plants *V. faba* and *L. edulis* were cultivated near the village of Zaros on the island of Crete (Greece). After harvesting (2005), the aerial parts (leaves and branches) were left to dry in the shade at room temperature and were then pulverized by a mill machine. The detailed experimental procedures of methanolic extraction, fractionation, and identification of flavonoid compounds were previously described.[33]

Wheat Germ Topoisomerase I Catalytic Assay. The standard wheat germ topoisomerase I reaction mixture (20 μL) contained 50 mM Tris-HCl (pH 7.5), 20% glycerol, 50 mM NaCl, 0.1 mM EDTA, 1 mM DiThioThreitol (DTT), 1 μg of Bluescript SK+, and 1 unit of enzyme (1 unit is defined as the amount of enzyme required to convert 90% of 1 μg of supercoiled DNA substrate into the relaxed, open circular conformation under the standard assay conditions). Reactions were carried out at 37 °C for 45 min.

Human Topoisomerase I Catalytic Assay. The standard human topoisomerase I reaction mixture (20 μL) contained 10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol, 0.25 μg of Bluescript SK+, and 1 unit of human topoisomerase I (1 unit is defined as the amount of enzyme required to convert 90% of 0.25 μg of supercoiled DNA substrate into the relaxed, open circular conformation under the standard assay conditions). Human topoisomerase I dilutions were performed in 10 mM Tris-Cl (pH 7.5), 500 mM NaCl, 1 mM PMSF, 2 mM DTT, and 50 μg/mL BSA. Reactions were carried out at 37 °C for 45 min.

Human Topoisomerase II Catalytic Assay. The standard human topoisomerase II reaction mixture (20 μL) contained 50 mM Tris-HCl (pH 8), 150 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM DTT, 30 μg of BSA/mL, 0.2 μg of Bluescript SK+, and 1.6 units of human topoisomerase II (1.6 units is defined as the amount of enzyme required to relax 90% of 0.2 μg of supercoiled DNA substrate under the standard assay conditions). Human topoisomerase II dilutions were performed in 10 mM Tris-Cl (pH 7.5), 500 mM NaCl, 1 mM PMSF, 2 mM DTT, and 50 μg/mL BSA. Reactions were carried out at 37 °C for 1 h.

Electrophoresis, Quantitation, and Statistical Analysis. All reactions were terminated by adding 5 μL of loading buffer (0.25% bromophenol blue and 30% glycerol). Electrophoresis was conducted on 0.8% agarose gel in TBE buffer (10 mM Tris-Cl, 90 mM boric acid, 0.5 M EDTA, pH 8) and run at 70 V for 2 h. The gels were stained with 0.5 μg/mL ethidium bromide (30 min), destained with H_2O (30 min), photographed by UV translumination using Alpha View software (Alpha Innotech, San Leandro, CA, USA), and analyzed with Gel-Pro Analyzer version 3.0 (MediaCybernetics, Silver Spring, MD, USA).

The percentage inhibition of topoisomerase-induced DNA strand cleavage by the tested compounds was calculated using the equation

$$\%\ \text{inhibition} = \frac{S_p - S}{S_p - S_o} \times 100$$

where S_o is the percentage of supercoiled conformation in the negative control sample (plasmid DNA), S_p is the percentage of supercoiled conformation in the positive control sample (plasmid DNA with topoisomerase), and S is the percentage of supercoiled conformation in the sample containing plasmid DNA, topoisomerase, and the tested compound.

It should be noted that isolated Bluescript SK+ plasmid DNA contained approximately 10–20% open circular DNA prior to treatment. Methanolic red grape extract (Mandilaria variety) was used as a marker of a potent topoisomerase I inhibitor as previously demonstrated.[44] Additionally, Bluescript SK+ plasmid DNA was treated with each compound alone at the highest concentration used (1000 μM for wheat germ topoisomerase I, 500 μM for human topoisomerase I, and 1000 μM for human topoisomerase II) to test its effects on plasmid DNA conformation. The inhibitory efficiency of the tested compounds was evaluated by their IC_{50} values, which represent the compound concentration needed to inhibit by 50% the relaxation of the supercoiled conformation induced by topoisomerases I and II.

Each experiment was carried out in triplicate. The results are expressed as mean ± SEM. Statistical computations were carried out using the SPSS 13.0 software. For statistical analysis, one-way ANOVA was applied, followed by Dunnett's test for multiple pairwise comparisons. Dose–response relationships were examined by Spearman's correlation analysis. Differences were considered to be significant at $p < 0.05$.

Cell Cultures and Cytotoxicity Measurements. Human breast adenocarcinoma cell line MCF7, human cervical carcinoma cell line HeLa, and human hepatocellular liver carcinoma cell line HepG2 were obtained from the Theageneio Anticancer Hospital of Thessaloniki, Greece, by serial subculturing. All cell cultures were maintained in DMEM (Dulbecco's modified Eagle medium, Gibco) (4500 mg/L D-glucose, 110 mg/L sodium pyruvate, L-glutamine) supplemented with 10% heat-inactivated FBS (fetal bovine serum, Gibco) and PS (100 units/mL of penicillin G, 100 μg/mL of streptomycin sulfate) (Gibco), in a humidified chamber at 37 °C, 5% CO_2.

Cytotoxicity was measured by the previously reported XTT assay method.[64] In brief, seeding density for HeLa, MCF7, and HepG2 was 1.5×10^4, 2×10^4, and 3.5×10^4 cells/well, respectively, in culture medium (DMEM−FBS−PS) in a 96-well flat-bottomed microplate. After 24 h incubation at 37 °C under humidified 5% CO_2, test solutions in DMEM-PS medium were added and incubated for an additional 48 h. Cultures containing 100 μM etoposide (Sigma) were used as positive control, while cultures containing only DMEM−PS were used as negative control. Compound **2** was added in cultures at concentrations of 100, 200, 300, 350, 400, and 500 μM. Cell cultures were additionally incubated for 4 h with the XTT reagent (Roche), and the optical density (OD) was measured using an EL808 microplate reader (Biotek Instruments, Inc.), at 450 nm with a reference wavelength at 650 nm. Furthermore, the OD of compound **2** (incubated with XTT reagent, without cells) at the tested concentrations was evaluated at both wavelengths. The percentage of cell survival (viability) was calculated as follows:

$$\text{viability} = \frac{[\text{OD(treated cells with } \mathbf{2}) - \text{OD(} \mathbf{2} \text{ alone)}]}{\text{OD(negative control cells)}}$$

Finally the IC_{50} values (concentration of compound **2** required to produce 50% inhibition of cell growth) were calculated for each cell line. At least four independent experiments were performed in duplicates. The results are expressed as mean ± SEM. Statistical computations were performed as described in the previous section.

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