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Antioxidant and Chemopreventive Properties of Polyphenolic Compounds Derived from Greek Legume Plant Extracts

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Recently, phytochemical compounds present in legumes have gained a lot of interest because they are considered to be possible chemopreventive agents. In the present study, 14 polyphenolic compounds were extracted and identified from two unique varieties of Leguminosae family plants cultivated in Greece and screened for their antioxidant and chemopreventive properties. Ten polyphenolic fractions, which are mainly mixtures of two compounds and five pure flavonoids, were isolated from the methanolic extracts of aerial plant parts of *Vicia faba* and *Lotus edulis* (Leguminosae), respectively. All of these fractions exhibited significant DPPH$^{\bullet}$ radical scavenging capacity. Furthermore, they exerted significant protective activity against free radical-induced DNA damage. This activity was more potent against ROO$^{\bullet}$ radical-induced DNA damage than against that induced by OH$^{\bullet}$ radicals. Finally, they exhibited significant ability to inhibit the activity of the topoisomerase I enzyme. These results imply that the polyphenolic compounds identified in the fractions were responsible of the observed properties of the fractions and the initial extracts and indicate different mechanisms by which these phenolic compounds may act as chemopreventive agents.

KEYWORDS: Legumes; flavonoids; DNA damage; antioxidants; ROS; chemoprevention; phytochemicals; *Vicia faba*; *Lotus edulis*

INTRODUCTION

In recent years, a lot of research has been done in the development of chemopreventive agents derived from foods that constitute integral parts of the human diet (*1–3*). Legumes, which play a crucial role in many diets worldwide, are thought to be related with beneficial heath implications in chronic diseases such as certain cancer types (colon, breast, prostate) (*4*), cardiovascular diseases (*5*), and diabetes (*6*). Except from their known high nutritive value (*7*), significant quantities of phytochemical compounds are identified in legumes and considered to be responsible for their beneficial effects (*8–10*). Phenolic compounds such as phenolic acids, lignans, and flavonoids (mainly isoflavonoids) present in legumes and other edible plant sources have been proposed to exert chemopreventive actions through various mechanisms (*11–15*). The heterogeneity in the varieties of legumes and their complicated and different phytochemical compositions make important the need for further research on the bioactive compounds present in legumes and their biological properties.

Because the mechanisms by which polyphenolic compounds present in legumes exert their biological properties have now been started to be evaluated, the aim of the present study was to examine the possible antioxidant and chemopreventive properties of some polyphenolic compounds isolated from two specific varieties of Leguminosae family plants cultivated in Greece. In a previous study, we examined the antiradical and protective activities against free radical-induced DNA damage of methanolic and aqueous extracts derived from various unique Greek Leguminosae family plants (*16*). The antioxidant and protective properties against free radical-induced DNA damage were attributed to the bioactive compounds present in them (*16*). Thus, in extending this study, methanolic extracts from the aerial parts of the edible herbs *Vicia faba* and *Lotus edulis* were chosen for further investigation. All of the isolates derived from the former extracts were screened for their possible chemopreventive abilities using different in vitro assays.

Because free radicals are considered to be involved in many ways in the multistage carcinogenic process (*17*), the antioxidant capacity of the isolates was first assessed using the DPPH radical

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scavenging assay. Second, to examine their protective-antimutagenic activity against free radical-induced DNA damage, peroxyl ($ROO^{\bullet}$) and hydroxyl ($OH^{\bullet}$) radical-induced DNA strand scission assays were used. Afterward, all of the isolates were assessed for their ability to inhibit the activity of the topoisomerase I enzyme, using the topoisomerase I relaxation assay. Topoisomerase I is one of the enzymes playing a crucial role in replication, transcription, recombination, chromosome condensation, and maintenance of genome stability (*18*). Because it is an essential enzyme for vital functions of DNA during normal cell growth, inhibitors of its activity are considered to be promising anticancer agents (*19*).

MATERIALS AND METHODS

Chemicals and Reagents. 2,2′-Azobis(2-amidinopropane hydrochloride) (AAPH) and 1,1-diphenyl-2-picrhydrazyl ($DPPH^{\bullet}$) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (H_2O_2) was purchased from Merck (Darmstadt, Germany), and topoisomerase I from wheat germ was obtained from Promega (Madison, WI). Bluescript-SK+ plasmid DNA was isolated from a large-scale bacterial culture. The other chemicals and solvents used were of the highest quality commercially available.

Plant Material. The edible plants *V. faba* and *L. edulis* (Leguminosae) were cultivated in a parcel near the village of Zaros on the island of Crete (Greece). After harvesting (2005), the aerial parts of the plants (leaves and branches) were transferred to the laboratory and left to dry under shadow at room temperature. Finally, the air-dried plant material was pulverized by a mill machine (Allenwest, Brighton, U.K.).

General Experimental Procedures of Fractionation and Identification. Thin-layer chromatography (TLC) was carried out on glass precoated silica gel 60 F_{254} plates (Merck). Fast centrifugal partition chromatography (FCPC) was performed using a CPC KROMATON with a 1000 mL column, adjustable rotation of 200−2000 rpm, and a Laboratory Alliance pump with a pressure safety limit of 50 bar. A manual sample injection valve was used to introduce the samples into the column. A Thermo Finnigan HPLC instrument was employed making use of a SpectraSystem P4000 pump, a SpectraSystem 1000 degasser, a SpectraSystem AS3000 automated injector, and a UV SpectralSystem UV2000 detector. NMR spectra were obtained with Bruker AC200 and Bruker DRX 400 spectrometers. Chemical shifts are given as δ values with TMS as the internal standard. The 2D experiments (COSY, COSY LR, HMQC, HMBC, TOCSY, and NOESY) were performed using standard Brucker microprograms. ESMS spectra were recorded with a Nermag R 10 10C apparatus.

Extraction, Fractionation, and Identification of Flavonoid Isolates. The powder of the aerial parts of *V. faba* (0.890 kg) and *L. edulis* (0.490 kg) was extracted with CH_2Cl_2 (dichloromethane), MeOH (methanol), and H_2O (water), successively. Each solvent extraction (3 × 5 L) was repeated three times, for 48 h per extraction. The MeOH extract of *V. faba* (48.1 g) was processed with resin XAD-4 to separate the total of phenolic compounds (12.4 g). A part of this fraction (12 g) was subjected to CPC separation using a mixture of EtOAc/*n*-BuOH/H_2O (2:1:3) as biphasic system. The separation was run at a revolution speed of 1000 rpm. Initially, the lower phase was used as the mobile phase (water based), whereas the upper phase was used as the stationary phase in a head to tail or descending mode (flow rate = 15 mL/min). The effluent of the column was manually collected in 30 mL aliquots, and this procedure resulted in 11 fractions (Vf A−L). Then, the organic layer in a tail to head or ascending mode and five fractions (Vf M−Q) were collected.

Similarly, a part of the *L. edulis* MeOH extract (12 g) was subjected to CPC separation using a mixture of heptane/EtOAc/MeOH/H_2O (1:4:1:4) as biphasic system. The separation was run at a revolution speed of 1100 rpm. Initially, the upper layer of the biphasic system was used as the mobile phase, whereas the lower layer (water based) was used as the stationary phase in a tail to head or ascending mode (flow rate = 15 mL/min). The effluent of the column was manually collected in 30 mL aliquots, and this procedure resulted in nine fractions (Le A−I). Then, the water layer was used as mobile phase in a head to tail or descending mode, and six fractions (Le K−P) were collected.

All fractions isolated from both plants were examined by TLC and analytical high-performance liquid chromatography (HPLC) using a 250 × 4.0 mm i.d. Lichrosorb RP18 column (BIRSHOFF Chromatography, Loenberg, Germany). The mobile phase was a linear gradient of 2% aqueous acetic acid (solvent A) and acetonitrile (solvent B). Conditions: initial A/B (95:5); in 70 min A/B (75:25); back to initial conditions; flow rate = 1 mL/min. Further purification of the compounds of interest was accomplished using preparative HPLC. All compounds were structurally elucidated using 1D and 2D NMR spectroscopy as well as UV and MS techniques. All of the known compounds were confirmed by comparison to literature data.

DPPH Radical Scavenging Assay. Free radical scavenging capacity was evaluated on the basis of the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$), measuring the reduction of absorbance at 517 nm (*20*). Briefly, the reaction was carried out in 1 mL of methanol containing 100 μM freshly made $DPPH^{\bullet}$ in methanol and the tested isolates at different concentrations (5−1000 μg/mL). The contents were vigorously mixed and incubated at room temperature in the dark for 20 min, and the absorbance was read at 517 nm using a Hitachi U-1500 spectrophotometer (San Jose, CA). In each experiment, the tested sample alone in methanol was used as blank and $DPPH^{\bullet}$ alone in methanol was used as control. All experiments were carried out in triplicate and at least on two separate occasions. The radical scavenging capacity (RSC) of the tested extracts was expressed as the percentage of $DPPH^{\bullet}$ elimination calculated according to the following equation:

$$\%\ \mathrm{RSC} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (1)$$

Moreover, to compare the radical scavenging efficiency of the samples, IC_{50} was also evaluated, showing the concentration of the tested isolate that has the ability to scavenge $DPPH^{\bullet}$ radical by 50%.

Peroxyl Radical-Induced DNA Strand Scission Assay. The assay was performed using the procedure of Chang et al. (*21*). Peroxyl radicals were generated from thermal decomposition of 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH). The reaction mixture (10 μL) containing 1 μg of Bluescript-SK+ plasmid DNA, 2.5 mM AAPH in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), and the tested sample at different concentrations (1, 2, 5, 10, 20, 50 μg/mL) was incubated in the dark for 45 min at 37 °C. AAPH was added at the end right before incubation. The reaction was terminated by the addition of 3 μL of loading buffer (0.25% bromophenol blue and 30% glycerol) and analyzed in 0.8% agarose gel electrophoresis at 70 V for 1 h. The gels were stained with ethidium bromide 0.5 μg/mL, destained with water, photographed by UV translumination using the Vilber Lourmat photodocumentation system (DP-001.FDC) (Torcy, France), and analyzed with Gel-Pro Analyzer version 3.0 (MediaCybernetics, Silver Spring, MD). Each experiment was carried out in triplicate.

Hydroxyl Radical-Induced DNA Strand Scission Assay. Hydroxyl radical-induced plasmid DNA relaxation assay was performed according to the method of Keum et al. (*22*) with some modifications. Hydroxyl radicals ($OH^{\bullet}$) were generated from UV photolysis of hydrogen peroxide (H_2O_2). The reaction mixture (10 μL) consisted of 1 μg of Bluescript-SK+ plasmid DNA, 10 mM Tris-HCl, 1 mM EDTA, the tested sample at different concentrations (100, 200, 400, 800, 1600 μg/mL), and 40 mM H_2O_2. Immediately after the addition of H_2O_2, the reaction mixture was irradiated with a 300 W UV lamp (OSRAM) for 3 min at a distance of 50 cm. The reaction was terminated by the addition of 3 μL of loading buffer (0.25% bromophenol blue and 30% glycerol) and analyzed in gel electrophoresis as described previously. Each experiment was carried out in triplicate. Additionally, Bluescript-SK+ plasmid DNA was also treated with each isolate alone at the highest concentration used (1600 μg/mL) to test its effects on plasmid DNA conformation.

Inhibition of Free Radical-Induced DNA Damage. Induction of DNA strand breaks by peroxyl ($ROO^{\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) was measured by the conversion of supercoiled Bluescript-SK+ plasmid

double-stranded DNA to open circular conformation analyzed in agarose gel electrophoresis. Preventive activity of the tested samples was assessed by inhibition of conversion of supercoiled (unnicked) conformation to open circular (nicked). The percentage inhibition of radical-induced DNA strand cleavage by the tested isolates was calculated using the equation

$$\% \text{ inhibition} = \frac{S_p - S}{S_p - S_0} \times 100 \quad (2)$$

where S_0 is the percentage of supercoiled conformation in the negative control sample (plasmid DNA alone), S_p is the percentage of supercoiled conformation in the positive control sample (plasmid DNA with the radical initiating factor), and S is the percentage of supercoiled conformation in the sample containing plasmid DNA, the tested isolate, and the radical initiating factor. It should be also noted that isolated Bluescript-SK+ plasmid DNA contained approximately 10−20% open circular DNA prior to treatment. Moreover, to compare the efficiency of preventive capacities of the tested isolates, IC_{50} was evaluated showing the concentration needed to inhibit relaxation of supercoiled conformation induced by $ROO^{\bullet}$ and $OH^{\bullet}$ radicals, respectively, by 50%.

Topoisomerase I Relaxation Assay. DNA topoisomerase I was assayed by measuring the decreased mobility of the relaxed conformation of supercoiled Bluesctript-SK+ plasmid DNA in an agarose gel. The standard topoisomerase I mixture (20 μL) contained 50 mM Tris-HCl (pH 7.5), 20% glycerol, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 μg of Bluescript SK+, and 1.3 units of enzyme (1.3 units 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 15 min and then terminated by adding 5 μL of loading buffer (0.25% bromophenol blue and 30% glycerol) and analyzed in 0.8% gel electrophoresis at 70 V for 1 h. Further analysis of gels was performed as described previously. In this assay, aqueous red grape extract (Mandilaria variety) was used as a marker of a very potent topoisomerase I inhibitor by showing 89% inhibitory activity at 200 μg/mL (*23*). Each experiment was carried out in triplicate, and the percentage of inhibition of the samples in the relaxation activity of topoisomerase I was calculated using eq 2, where S_p is the percentage of supercoiled conformation in the positive control sample (plasmid DNA with 1.3 units of enzyme) and S is the percentage of supercoiled conformation in the sample containing plasmid DNA, the tested isolate (20, 50, 100, 200 μg/mL), and 1.3 units of enzyme, respectively.

Statistical Analysis. All results are expressed as mean ± SD ($n = 3$). Statistical computations were carried out using 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 $p < 0.05$.

RESULTS AND DISCUSSION

Flavonoid Fractions Isolated from the Legume Plant Extracts. Ten fractions isolated from the aerial parts extract of *V. faba* and four from the extract of *L. edulis* were chosen for further evaluation of their antioxidant and chemopreventive abilities. The composition of *V. faba* fractions was as follows: Vf B (0.320 g), **1**:**2**:**3** (3:1:3); Vf C (0.320 g), **1**:**2** (2:1); Vf E (0.235 g), **3**:**4** (3:1); Vf F (0.438 g), **3**:**4** (2:1); Vf I (0.305 g), **5**:**6** (1:4); Vf K (0.245 g), **5**:**10**:**6** (4:1:2); Vf L (1.123 g), mixture of nonpolar compounds; Vf N (0.291 g), **7**:**8** (2:1); Vf P (0.800 g), **9**:**10** (1:1); Vf Q (0.841 g), **5**:**10** (2:3). The structures of the compounds that constitute each fraction are depicted in **Figure 1**. From the polyphenolic compounds identified in the fractions the majority were kaempferol and quercetin glycosides (**Figure 1**). To our knowledge, there are no recent data about this type of flavonoid present in the aerial parts of *V. faba* plants, although proanthocyanidins have been previously isolated and identified in *V. faba* seed coats (*24*). Similarly, the composition of *L. edulis* fractions was as follows: Le B (0.920 g), **13**; Le E (0.420 g), isomers of **11** (1:1); Le N (0.940 g), **7**; Le O (0.460 g), **12**. On the contrary, with *V. faba* fractions, which consisted of a combination of phenolic compounds, *L. edulis* fractions mainly consisted of pure flavonoid compounds (**Figure 2**). Only the Le E fraction consisted of a mixture of two isomers of compound **11**. As it is obvious from their chemical structures, they constitute kaempferol glycosides (**Figure 2**). All of the known compounds (**1**−**11**) were confirmed by comparison to literature data. The fact that *V. faba* and *L. edulis* are two distinct plants explains the differences observed in the composition of polyphenolic compounds present in them. The kaempferol glycosides identified were isolated in the *L. edulis* aerial plant part for the first time. However, similar flavonoid glycosides have been previously identified in *Lotus polyphyllos*, another member of the *Lotus* genus of the Leguminosae family of plants (*25*). In previous studies, the majority of phytochemical compounds identified and screened for their biological properties were derived from seeds and seed coats of other legumes, especially from varieties of *Phaseolus vulgaris* (*26, 27*).

The formulas of compounds **12** and **13** are tentative, and their spectroscopic data are presented in **Tables 1** and **2**, respectively. Identification of compounds **12** and **13** was performed by 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, HMBC, HMQC) NMR and MS experiments. The ^{1}H NMR of **12** (**Table 1**) exhibited A and B ring signals typical of kaempferol 3,7-di-*O*-substituted glycosides (*28*). In more detail, this spectrum showed two pairs of doublets, one at δ 7.90 (2H, d, $J = 8.2$ Hz), which was assigned to H-2′ and H-6′, and the other at δ 6.94 (2H, d, $J = 8.2$ Hz), which was assigned to H-3′ and H-5′. The two protons resonating at δ 6.73 and 6.46 (br s), each with a one-proton integration, were assigned for C-8 and C-6, respectively. This downfield chemical shift confirmed that C-7 is substituted in ring A of the compound (*29*). The presence of two anomeric protons at δ 5.73 (br s) and 5.57 (br s) indicated that the two sugar units are attached to the aglycone. The first of them corresponds to a β-D-apiofuranosyl moiety, as was supported by the chemical shift difference of 2.9 ppm observed for C-2″ and C-3″ (*30*). The second anomeric signal and the methyl group at δ 1.28 (d, $J = 6.5$ Hz) indicated the presence of a rhamnose unit, which is attached to the aglycone through an α-linkage (*31*).

The ^{13}C NMR (**Table 2**) and DEPT experiments of compound **12** showed the presence of 2 methylene, 1 methyl, 13 methine, and 10 quaternary carbons. Fifteen of these signals were assigned to kaempferol as the aglycon moiety, 6 to the rhamnose unit, and 5 to the apiose moiety. The downfield chemical shift of the C-4 (δ 179.5) indicated the presence of a chelated hydroxyl function at C-5, which was also in accord with the chemical shift of C-5 (δ 162.6). In the HMBC spectrum was obvious a correlation between the anomeric proton of the rhamnopyranosyl unit (H-1‴, δ 5.57) and C-7 (δ 163.5), although the anomeric proton of the apiosyl unit (H-1″, δ 5.73) showed a correlation with C-3 (δ 135.2). The assignments of the various sugar protons were made by their spin-pattern analysis, COSY, and HMQC experiments. These results confirmed the new structure of compound **12** as kaempferol 3-*O*-apiofuranosyl-7-*O*-rhamnopyranosyl.

The NMR data of compound **13** were similar to those of compound **12**. However, the signals at δ 2.10 (3H, s) in the ^{1}H NMR and δ 20.7 and 172.6 in the ^{13}C NMR spectra of **13** (**Table 2**) revealed the presence of an acetyl group. This moiety was assigned to the C-5‴ on the basis of the observed downfield shift of H-5‴ (δ 4.18 and 4.06 instead of δ 4.14 and 3.89 for

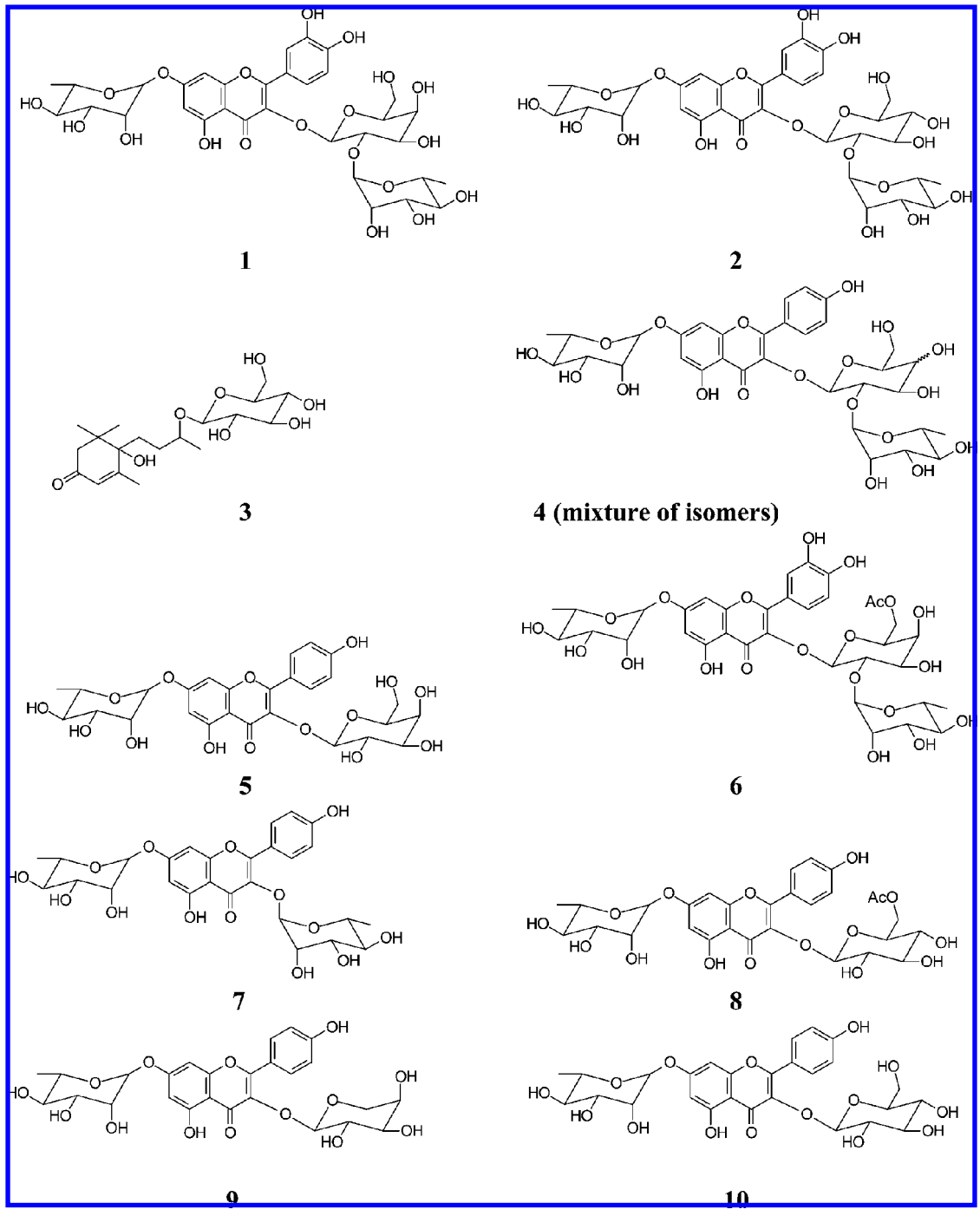

Figure 1. Compounds identified in the polyphenolic fractions isolated from *V. faba* methanolic plant extract. Compounds: **1**, quercetin 3-*O*-rhamnopyranosyl(1→2)-galactopyranoside-7-*O*-rhamnopyranoside; **2**, quercetin 3-*O*-rhamnopyranosyl(1→2)-glucopyranoside-7-*O*-rhamnopyranoside; **3**, 9-*O*-β-D-glycopyranosyloxy-6-hydroxy-3-oxo-α-ionol; **4**, kaempferol 3-*O*-rhamnopyranosyl(1→2)-galactopyranoside-7-*O*-rhamnopyranoside and kaempferol 3-*O*-rhamnopyranosyl(1→2)-glucoopyranoside-7-*O*-rhamnopyranoside; **5**, kaempferol 3-*O*-galactopyranoside-7-*O*-rhamnopyranoside; **6**, quercetin 3-*O*-rhamnopyranosyl(1→2)-6-*O*-acetylgalactopyranoside-7-*O*-rhamnopyranoside; **7**, kaempferol 3,7-di-*O*-rhamnopyranoside; **8**, kaempferol 3-*O*-(6-*O*-acetyl-glucopyranoside)-7-*O*-rhamnopyranoside; **9**, kaempferol 3-*O*-arabinoside-7-*O*-rhamnopyranoside; **10**, kaempferol 3-*O*-glycopyranoside-7-*O*-rhamnopyranoside.

compound **12**). The correlation between acetyl C═O and both of the apiosyl H-5‴ protons by HMBC confirms the previous assignment. ESMS positive at *m/z* 565 $[M + H]^+$ for **12** and *m/z* 607 $[M + H]^+$ for **13** established the molecular ion formulas $C_{26}H_{28}O_{14}$ and $C_{28}H_{30}O_{15}$, respectively.

Radical Scavenging Capacity of the Flavonoid Isolates. One of the most common assays used for the assessment of the antioxidant capacity of phenolic compounds in foods is DPPH radical scavenging assay (*32*). According to IC_{50} values (**Figure 3**), like the initial plant extracts, all of the polyphenolic fractions exerted significant abilities to scavenge DPPH$^\bullet$ radical. The values of IC_{50} indicating the radical scavenging efficiency of the polyphenolic fractions are presented in **Figure 3**. The lower the IC_{50} value, the higher the antioxidant capacity of the fraction. In detail, all polyphenolic fractions derived from the methanolic extract of aerial parts of *V. faba* were effective scavengers of the DPPH$^\bullet$ radical. IC_{50} data range from 30 to 198 μg/mL. According to the results Vf C, E, B, P, and Q polyphenolic

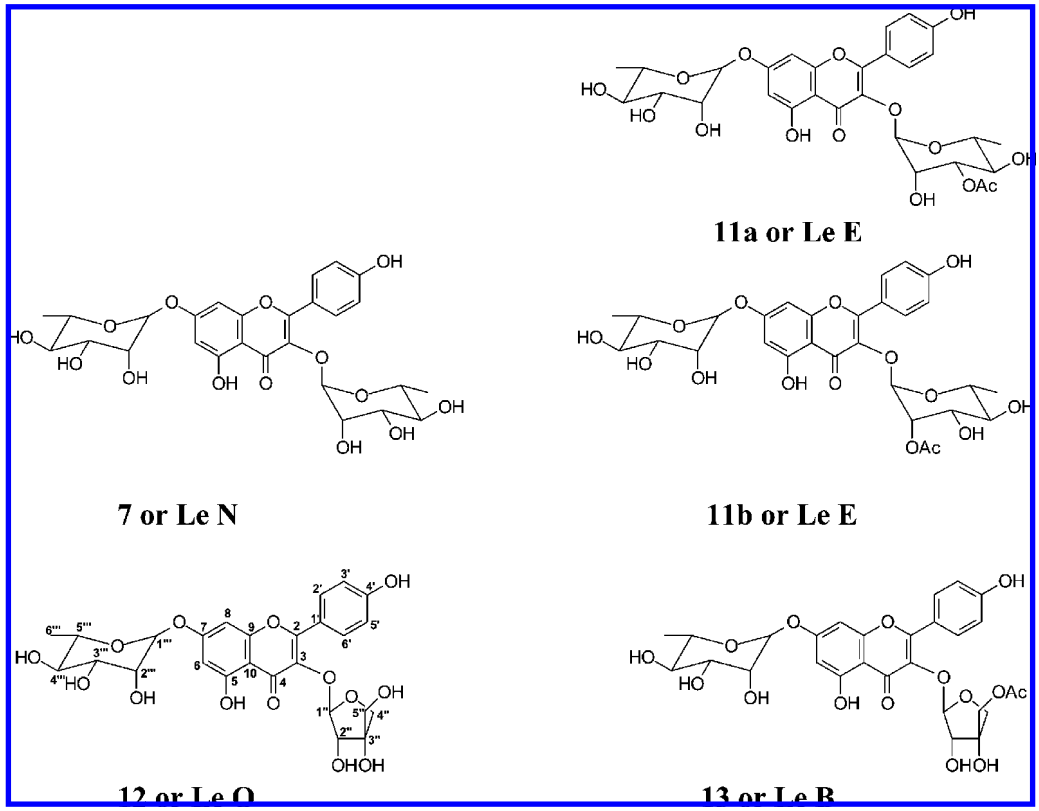

Figure 2. Flavonoids isolated from *L. edulis* plant extract. Compounds: **7** or Le N, kaempferol 3,7-di-*O*-rhamnopyranosyl; **11** or Le E, (**a**) kaempferol 3-*O*-(3-*O*-acetylrhamnopyranosyl)-7-*O*-rhamnopyranosyl or (**b**) kaempferol 3-*O*-(2-*O*-acetylrhamnopyranosyl)-7-*O*-rhamnopyranosyl; **12** or Le O, kaempferol 3-*O*-apiofuranosyl-7-*O*-rhamnopyranosyl; **13** or Le B, kaempferol 3-*O*-(5-*O*-acetylapiosyl)-7-*O*-rhamnopyranosyl.

Table 1. NMR Spectroscopic Data of Compound **12**

	^{1}H		^{13}C
C/H	δ	m, *J* (Hz)	δ
1			
2			159.6
3			135.2
4			179.5
5			162.6
6	6.46	br s	99.6
7			163.2
8	6.73	br s	95.3
9			157.7
10			107.1
1′			122.3
2′	7.90	d, 8.2	131.6
3′	6.94	d, 8.2	116.1
4′			161.3
5′	6.94	d, 8.2	116.1
6′	7.90	d, 8.2	131.6
1″	5.73	br s	110.9
2″	4.31	br s	
3″			79.2
4″α	3.54	d 12.5	76.4
4″β	3.64	d 12.5	
5″α	4.14	d 11.0	69.8
5″β	3.89	d 11.0	
1‴	5.57	br s	100.3
2‴	4.06	br s	72.0
3‴	3.86	dd 9.5, 1.7	72.5
4‴	3.52	t 9.5	73.7
5‴	3.64	m	71.7
6‴	1.29	d, 6.4	17.8

Table 2. NMR Spectroscopic Data of Compound **13**

	^{1}H		^{13}C
C/H	δ	m, *J* (Hz)	δ
1			
2			160.2
3			135.4
4			179.7
5			163.0
6	6.45	br s	99.9
7			163.6
8	6.74	br s	95.7
9			158.1
10			107.8
1′			122.6
2′	7.87	d, 8.2	132.1
3′	6.94	d, 8.2	116.5
4′			161.7
5′	6.94	d, 8.2	116.5
6′	7.87	d, 8.2	132.1
1″	5.72	br s	110.9
2″	4.23	br s	79.5
3″			76.6
4″a	3.53	d 12.5	79.2
4″b	3.64	d 12.5	
5″α	4.18	d 11.0	67.8
5″β	4.06	d 11.0	
1‴	5.58	br s	100.6
2‴	4.04	br s	71.2
3‴	3.85	dd 9.5, 1.7	71.7
4‴	3.50	t 9.5	73.6
5‴	3.64	m	71.7
6‴	1.28	d, 6.5	18.2
CH_3CO-	2.10	s	20.7
CH_3CO-			172.6

fractions were the most potent antioxidants, scavenging DPPH$^\bullet$ radical by 50% at 30−37 μg/mL. In contrast, Vf L, N, and K were the less potent DPPH$^\bullet$ radical scavengers. In comparison with the methanolic aerial plant part extract of *V. faba*, all of the polyphenolic fractions showed higher radical scavenging capacity than the extract (**Figure 3**).

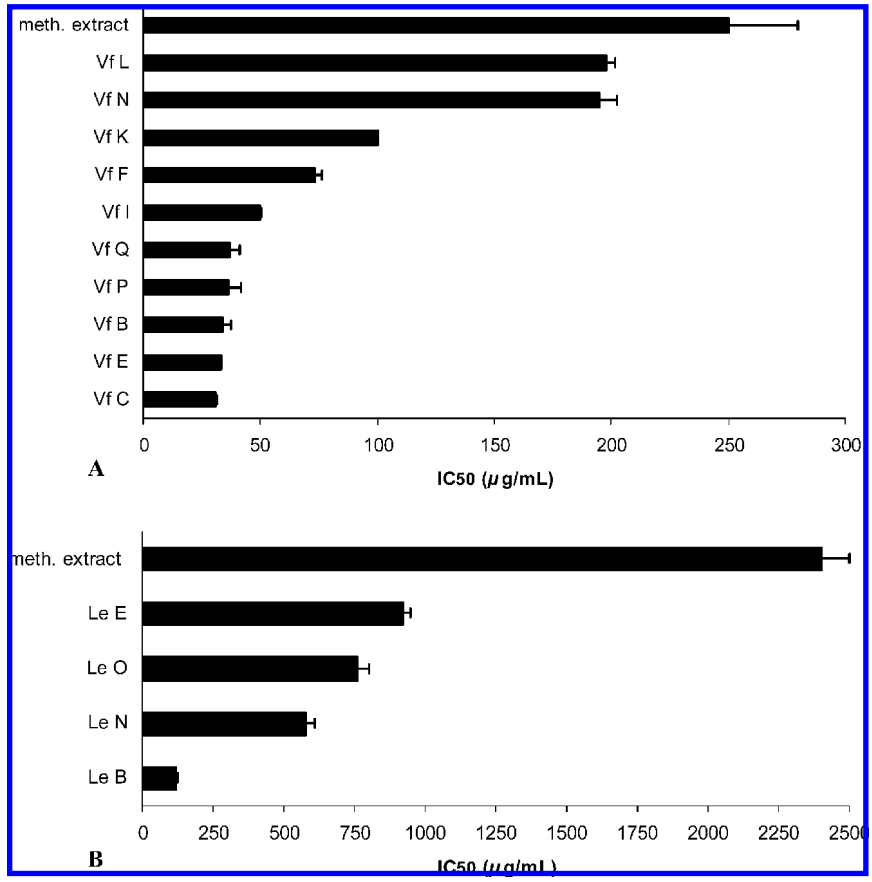

Figure 3. Radical scavenging capacity of polyphenolic isolates: (**A**) *V. faba* polyphenolic fractions; (**B**) *L. edulis* flavonoid fractions. Bars show means ± standard deviations of at least two separate triplicate experiments.

The pure flavonoids isolated from the methanolic extract of aerial parts of *L. edulis* were also efficient scavengers of DPPH$^{\bullet}$ radical, but they were less efficient than the polyphenolic fractions derived from *V. faba*. IC_{50} data range from 120 to 580 μg/mL. According to IC_{50} values, presented in **Figure 3**, Le B (compound **13**) showed the most potent and Le E (compound **11**) the least potent antioxidant activity. Among them, Le O (compound **12**), which differs from Le B in one acetyl group ($-COCH_3$) in its chemical structure, was a less potent radical scavenger than Le B. On the contrary, Le N (compound **7**), which also differs in one acetyl group ($-COCH_3$) from Le E, was a more potent radical scavenger than Le E. Finally, similarly with *V. faba* plant extract, the *L. edulis* plant extract also had a lower radical scavenging capacity than that of the pure flavonoids (**Figure 3**).

The results obtained indicate that these flavonoid glycosides may constitute some of the extract components responsible for the radical scavenging capacities of the extracts (*16*). Pure flavonol glycosides, similar to that identified in the fractions, have been previously reported to exert significant radical scavenging capacities, although their activity was weaker than that of their aglycone forms (*33, 34*). In previous studies, the polyphenolic compounds isolated from various bean extracts were also considered to be the compounds responsible for the observed antioxidant activity (*35*). It was also noteworthy that the radical scavenging capacity of the isolates was higher than that of the initial plant extract (**Figure 3**), implying that either the combination of the polyphenolic compounds in the extracts may not behave in the same way as they act when they are pure or their enrichment in the final preparation is the cause of the higher activity.

Furthermore, the different combinations of polyphenolic compounds in the fractions isolated from *V. faba* extract, along with the interactions that may occur between them, could explain the differences in the radical scavenging efficiencies observed between the fractions (**Figure 3**). It was also of interest that the pure flavonoids isolated from *L. edulis* plant extract, Le O, Le N, Le B, and Le E, showed different radical scavenging effects, although the only difference in their chemical structure was that Le O and Le N have one hydroxyl group replaced by an acetyl group ($-COCH_3$), in Le B and Le E, respectively (**Figure 1**). Because *L. edulis* fractions consist of single polyphenolic compounds, it was more feasible to correlate their structural characteristics with the observed properties. Generally, the interaction of a potential antioxidant with the DPPH$^{\bullet}$ radical depends on its structural conformation, depending on the number and availability of the hydroxyl groups (*36*), although Le B was a more potent radical scavenger than Le O, indicating a more complex mechanism of interaction of these compounds with DPPH$^{\bullet}$ radical. Additionally, the fact that the fraction Vf L was the only fraction consisting of nonpolar compounds may explain its low radical scavenging capacity (**Figure 3**).

Protective Activity of the Flavonoid Isolates against Free Radical-Induced DNA Damage. Because all of the polyphenolic fractions displayed significant antioxidant activity, they were also tested for their ability to prevent free radical-induced DNA damage. Thermal decomposition of AAPH and UV photolysis of H_2O_2 produces ROO$^{\bullet}$ and OH$^{\bullet}$ radicals, respectively, which cause DNA single-strand breaks resulting in the conversion of supercoiled conformation of plasmid DNA to open circular conformation. *V. faba* and *L. edulis* methanolic extracts of aerial plant parts exhibited significant protective activity

Table 3. Protective Activity of *V. faba* and *L. edulis* Polyphenolic Fractions against Peroxyl and Hydroxyl Radical-Induced DNA Damage

polyphenolic fractions *Vicia faba*	IC_{50}[a] (μg/mL) ROO$^\bullet$	IC_{50}[a] (μg/mL) OH$^\bullet$	flavonoid fractions *Lotus edulis*	IC_{50}[a] (μg/mL) ROO$^\bullet$	IC_{50}[a] (μg/mL) OH$^\bullet$
Vf P[b]	1.9 ± 0.1	653 ± 55.1	Le B[b]	9.8 ± 0.4	NT[c]
Vf C	2.1 ± 0.1	>1600[d]	Le E	13.3 ± 0.3	220 ± 34.6
Vf I	2.7 ± 0.3	420 ± 72.1	Le O	30.2 ± 5.3	249 ± 34.6
Vf E	2.8 ± 0.3	1157 ± 140.1	Le N	34.2 ± 3.0	1337 ± 32.1
Vf Q	3.0 ± 0.1	560 ± 40.0	meth total extract[e]	48.7 ± 6.5	>1600
Vf B	3.8 ± 0.2	>1600			
Vf F	5.5 ± 0.9	1010 ± 165.2			
Vf K	6.2 ± 0.4	450 ± 50.0			
Vf N	6.9 ± 0.4	293 ± 49.3			
Vf L	9.4 ± 1.2	NT			
meth total extract[f]	28.3 ± 2.7	>1600			

[a] Values are the mean ± SD from three independent experiments. [b] Polyphenolic fraction number. [c] NT, not tested isolate. [d] The sample could not reach 50% inhibition of DNA damage at the highest tested concentration (1600 μg/mL). [e] Methanolic extract of aerial parts of *L. edulis*. [f] Methanolic extract of aerial parts of *V. faba*.

against both ROO$^\bullet$ and OH$^\bullet$ radical-induced DNA damage (*16*). All of the tested isolates, derived from either plant extract, were also significant inhibitors of DNA damage induced by ROO$^\bullet$ and OH$^\bullet$ radicals (**Table 3**), supporting the hypothesis that these polyphenolic compounds constitute the extract components responsible for their DNA protective and antioxidant activity. All polyphenolic fractions derived from *V. faba* methanolic extract exhibited significant dose-dependent ($r > 0.88$, $p < 0.01$) protective activity against both ROO$^\bullet$ and OH$^\bullet$ radical-induced DNA damage. According to IC_{50} values, these fractions exerted more potent inhibitory activity against ROO$^\bullet$ radical-induced damage than against that induced by OH$^\bullet$ radicals, with IC_{50} values ranging from 1.9 to 9.4 μg/mL and from 293 to >1600 μg/mL, respectively (**Table 3**). However, the efficiency of the inhibitory activity of the fractions differs between the radicals. Characteristic were the results for Vf P, N, and B polyphenolic fractions. Briefly, the fraction Vf P, which was the most effective against the strand breaking activity of ROO$^\bullet$ radicals, was not so effective against that of OH$^\bullet$ radicals (**Table 3**). The Vf B fraction was similarly efficient against the activity of ROO$^\bullet$ radicals, whereas it showed only 25% inhibition at 1600 μg/mL against the activity of OH$^\bullet$ radicals. In contrast to Vf P and Vf B, the Vf N fraction was not so effective against the strand-breaking activity of ROO$^\bullet$ radicals, whereas it was the most effective against that of HO$^\bullet$ radicals (**Table 3**).

Pure flavonoids derived from *L. edulis* methanolic plant extract were also potent inhibitors of ROO$^\bullet$ and OH$^\bullet$ radical strand-breaking activity, although to a lesser extent in comparison with *V. faba* isolates. IC_{50} data values range from 9.8 to 34.2 μg/mL and from 220 to 1337 μg/mL for ROO$^\bullet$ and OH$^\bullet$ radical-induced DNA damage, respectively (**Table 3**). Among them, flavonoid Le E exhibited the most potent dose-dependent protective activity against both ROO$^\bullet$ ($r = 0.97$, $p < 0.01$) and OH$^\bullet$ radicals ($r = 0.95$, $p < 0.01$) (**Figure 4**). Le N, which differs from Le E only in an acetyl group, also inhibits dose dependently the activity of ROO$^\bullet$ ($r = 0.94$, $p < 0.01$) and OH$^\bullet$ radicals ($r = 0.96$, $p < 0.01$) (**Figure 4**), although it was less efficient than Le E (**Table 3**). Finally, similarly with the radical scavenging capacity, *V. faba* and *L. edulis* plant extracts had lower protective activity than their isolates (**Table 3**).

It was noteworthy that, similarly with the initial plant extracts, the protective activity of the isolates against the strand-breaking activity of OH$^\bullet$ radicals was not as high as that observed against the activity of ROO$^\bullet$ radicals (**Table 3**). The polyphenolic fractions from both extracts exerted significant inhibitory activity against ROO$^\bullet$ radical-induced conversion of supercoiled plasmid DNA conformation at concentrations ranging from 1 to 50 μg/mL, whereas they had no effect on OH$^\bullet$ radical DNA breakage at these concentrations. Moreover, polyphenolic fractions that were effective against ROO$^\bullet$ radicals were not so effective against OH$^\bullet$ radicals (**Table 3**). This result could be attributed to the fact that peroxyl and hydroxyl radicals constitute two distinct types of free radicals and indicates a different mechanism of interaction of these isolates with these radicals. Flavonols and their glycosides have been previously shown to inhibit LDL oxidation induced by ROO$^\bullet$ radicals generated from AAPH decomposition (*37*). Thus, the protective activity of the isolates against the DNA strand-breaking activity of ROO$^\bullet$ radicals, along with the fact that these radicals comprise one of the major factors initiating the cascade reactions of lipid peroxidation (*38*), implies that these isolates may prevent lipid peroxidation. Additionally, because both peroxyl radicals and lipid peroxidation can independently cause mutations on DNA (*39, 40*), which are known to be crucial for the initiation of the carcinogenic process, these polyphenolic isolates may be potential chemopreventive agents.

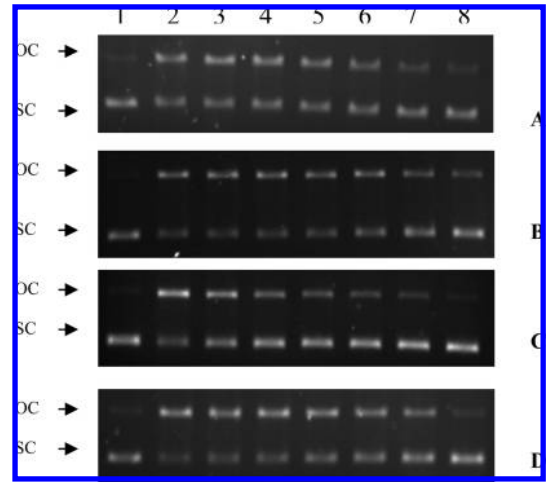

Figure 4. Protective activity of *L. edulis* flavonoid isolates on DNA strand scission induced by ROO$^\bullet$ and OH$^\bullet$ radicals: (**A**) Le E and (**B**) Le N activity against ROO$^\bullet$ radicals. Bluescript-SK+ plasmid DNA was exposed to ROO$^\bullet$ radicals alone (lane 2) or to ROO$^\bullet$ radicals in the presence of 1, 2, 5, 10, 20, or 50 μg/mL isolate, respectively (lanes 3−8). (**C**) Le E and (**D**) Le N activity against OH$^\bullet$ radicals. Bluescript-SK+ plasmid DNA was exposed to UV plus H_2O_2 (lane 2) or to UV plus H_2O_2 in the presence of 100, 200, 400, 800, or 1600 μg/mL isolate, respectively (lanes 3−7) or to 1600 μg/mL isolate alone (lane 8). Lane 1 represents Bluescript-SK+ plasmid DNA without any treatment. OC, open circular; SC, supercoiled.

Table 4. Inhibition of Catalytic Activity of Topoisomerase I by *V. faba* and *L. edulis* Aerial Plant Part Extracts and by Polyphenolic Fractions Derived from Them

plant polyphenolic isolate		% inhibition of Topo I activity at [a]			
		20 μg/mL	50 μg/mL	100 μg/mL	200 μg/mL
V. faba	Vf K	NI[b]	NI	NI	12 ± 2[c]
	Vf L	NI	NI	NI	21 ± 1[c]
	Vf N	NI	NI	9 ± 4	22 ± 1[c]
	Vf F	NI	10 ± 2	12 ± 0.3[c]	20 ± 0.4[c]
	Vf I	8 ± 2	13 ± 2	16 ± 2[c]	30 ± 3[c]
	Vf P	17 ± 2[c]	22 ± 2[c]	39 ± 3[c]	50 ± 2[c]
	Vf B	12 ± 2	28 ± 4[c]	39 ± 2[c]	51 ± 3[c]
	Vf C	30 ± 4[c]	39 ± 3[c]	39 ± 4[c]	53 ± 5[c]
	Vf E	71 ± 4[c]	75 ± 3[c]	76 ± 1[c]	79 ± 3[c]
	Vf Q	79 ± 3[c]	83 ± 3[c]	83 ± 4[c]	94 ± 6[c]
L. edulis	Le E	NI	NI	10 ± 1	26 ± 1[c]
	Le B	10 ± 2	22 ± 1[c]	28 ± 2[c]	61 ± 2[c]
	Le N	63 ± 5[c]	72 ± 4[c]	67 ± 1[c]	68 ± 5[c]
	Le O	66 ± 6[c]	69 ± 6[c]	71 ± 6[c]	72 ± 7[c]

plant extracts		% inhibition of Topo I activity at			
		100 μg/mL	200 μg/mL	400 μg/mL	800 μg/mL
V. faba	meth total extract	NI	NI	NI	21 ± 2[c]
L. edulis	meth total extract	NI	14 ± 4	20 ± 2[c]	24 ± 2[c]

[a] Values are the mean ± SE of % inhibition of Topo I activity in three independent experiments. [b] NI indicates no observed inhibition. [c] $p < 0.05$ when compared with positive control (DNA with 1.3 units of Topo I enzyme alone).

Furthermore, the observed protective activity against $OH^{\bullet}$ radicals, despite the fact that it was lower than that against $ROO^{\bullet}$ radicals, indicates an interaction with these radicals which constitute one of the most highly reactive oxygen species responsible for many DNA modifications (*41*). This result, considering the fact that solar UV radiation induces the production of $OH^{\bullet}$ radicals, also suggests these polyphenolic isolates as possible chemopreventive factors that can prevent the detrimental effects of UV radiation, which is considered to be the main factor responsible for several skin diseases including skin cancer (*42*).

It was noteworthy that none of the polyphenolic fractions alone affected plasmid DNA conformation. Moreover, the isolates Le B and Vf L were not tested by $HO^{\bullet}$ radical-induced DNA strand scission assay because they were dissolved in DMSO (16 and 4%, respectively), which at concentrations 1.2 and 0.8%, respectively, inhibited DNA strand-breaking activity of $OH^{\bullet}$ radicals (**Table 3**).

Inhibition of Topoisomerase I Plasmid Relaxation Activity. Finally, all of the fractions were tested for their ability to inhibit the catalytic activity, plasmid DNA relaxation activity, of the enzyme topoisomerase I (*43*). Topoisomerase I constitutes an essential enzyme for vital functions of DNA during normal cell growth, and its inhibitors are considered to be promising anticancer agents (*18, 19*). According to the results (**Table 4**), the majority of the fractions derived from both plant extracts exerted significant inhibitory abilities, with some of them inhibiting almost completely the catalytic activity of topoisomerase I even at the smallest tested concentration, 20 μg/mL. Vf K, L, and N fractions derived from *V. faba* plant extract exhibited the least potent inhibitory activity. These fractions exerted 12, 21, and 22% inhibition, respectively, only at the highest tested concentration, 200 μg/mL (**Table 4**). Among the rest of the polyphenolic fractions, Vf P, B, C, E, and Q exhibited the most significant inhibitory activity (**Figure 5** and **Table 4**).

Among the isolates derived from *L. edulis* extract, Le N and Le O were the most potent, inhibiting by 63 and 66%,

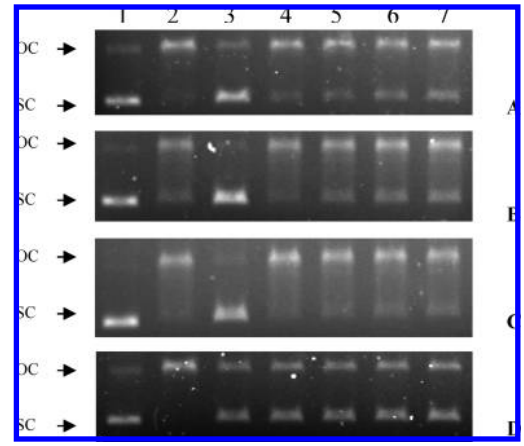

Figure 5. Inhibition of catalytic activity of topoisomerase I enzyme by polyphenolic isolates derived from *V. faba* and *L. edulis* plant extracts: (**A**) Vf P; (**B**) Vf B; (**C**) Le E; (**D**) Le N. Bluescript-SK+ plasmid DNA was relaxed by 1.3 units of topoisomerase I alone (lane 2) or along with the presence of 200 μg/mL aqueous red grape extract (lane 3) or with 20, 50, 100, or 200 μg/mL isolate, respectively (lanes 4−7). Lane 1 represents Bluescript-SK+ plasmid DNA without any treatment. OC, open circular; SC, supercoiled.

respectively, topoisomerase I at 20 μg/mL, the smallest tested concentration. In contrast, Le E and Le B were not so potent topoisomerase I inhibitors (**Figure 5** and **Table 4**). It was noteworthy that among flavonoid fractions derived from *L. edulis* plant extract, which consisted of pure flavonoid compounds, the flavonoids that lack acetyl groups in their structures were found to be the most potent (**Table 4**). This result indicates that the acetyl groups may interfere in the observed topoisomerase I inhibitory activity of the flavonoids (**Figure 5**). It was interesting also that fraction Vf N contains the same kaempferol derivative as fraction Le N, where it was pure. Vf N was more potent than Le N in the antioxidant assays used. On the contrary, Le N exhibited a more potent activity in the topoisomerase I relaxation assay. The presence of the other kaempferol derivative in fraction Vf N increases the antioxidant ability but reduces the ability to interfere with topoisomerase I. Furthermore, this result also implies a mechanism of interaction of this kaempferol derivative with topoisomerase I. In previous studies flavonoids and flavonoid glycosides have been identified as potent topoisomerase I inhibitors (*44, 45*). Thus, the potent inhibitory activity of these polyphenolic fractions could be another mechanism by which these compounds exert chemopreventive properties and broach them as a subject in anticancer drug design. It was noteworthy that DMSO at final concentrations 0.2 and 0.1% for Le B and Vf L isolates, respectively, had no effect on the catalytic activity of topoisomerase I.

Moreover, comparison of the data demonstrates that the topoisomerase I inhibitory activity of the isolates, similarly with their radical scavenging capacity, and their DNA protective activity were more potent than that observed for the initial plant extracts. Finally, all polyphenolic isolates exhibited more potent inhibitory activity than the initial methanolic plant extracts, which had the ability to inhibit plasmid relaxation activity of topoisomerase I at concentrations >400 μg/mL (**Table 4**). Briefly, *L. edulis* plant extract was more potent than *V. faba* plant extract by inhibiting topoisomerase I activity 20 and 24% at 400 and 800 μg/mL, respectively. *V. faba* methanolic extract exhibited 21% inhibition of topoisomerase I activity only at 800 μg/mL, the highest tested concentration.

In conclusion, this study reports significant antioxidant, DNA protecting, and topoisomerase I inhibitory properties of some

new polyphenolic isolates derived from two common Leguminosae family plants. *V. faba* fractions exhibited the most potent radical scavenging capacities and protective ability against free radical-induced DNA damage, whereas *L. edulis* fractions exhibited the most potent inhibitory activity on topoisomerase I enzyme activity. These properties indicate that these polyphenolic isolates can be considered to be possible chemopreventive agents. Because many chemopreventive agents act through more than one mechanism, further research must be done for the evaluation of these mechanisms and to clarify the chemopreventive properties in vivo.

ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl; $ROO^{\bullet}$, peroxyl radical; $OH^{\bullet}$, hydroxyl radical; AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); FCPC, fast centrifugal partition chromatography; RSC, radical scavenging capacity; Topo I, topoisomerase I.

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