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Chemistry and Bioactivity of Olive Biophenols in Some Antioxidant and Antiproliferative in Vitro Bioassays

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The major biophenols in olives and the crude extract and ethyl acetate fraction from olive mill waste were studied for their ability to counteract different stages of oxidative damage, that is, hydrogen peroxide, superoxide radical (SOR), and hydroxyl radical in vitro. Antiproliferative activity on colon cancer (HT-29) and gastric cancer (AGS) cell lines was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide bioassay. Emphasis was given to how the observed in vitro activity is controlled by the structural feature of biophenols and possible synergism and antagonism. While in some bioassays, for example, 2,2'-diphenyl-1-picrylhydrazyl, the nonphenolic moiety had minimal affect, it had a significant role in the SOR scavenging bioassay. Verbascoside was more active than caffeic acid or hydroxytyrosol evaluated individually or in equimolar mixtures in some bioassays. Mixtures of biophenols were more active than individual biophenols as antiproliferative agents. Overall, the mixture of hydroxytyrosol/caffeic acid and the biophenol extracts were more effective in protecting DNA from oxidative damage and inhibiting the growth of cancer cells.

Introduction

The nutritive and therapeutic virtues of olive products are attributed to their biophenolic contents (1–3). It is believed that the mechanism of action of these materials in relation to “prevention” of coronary heart diseases (4) and cancer (5) involves an antioxidant component either directly or indirectly. However, there is no general agreement on how to assess oxidative stress. The problem is not a shortage of bioassays (6, 7) but rather finding the one that correlates with long-term disease progression. Analogously, we do not have a standardized method for measuring therapeutic activity of antioxidants on the in vitro, ex vivo, or in vivo level. It is clear, however, that antioxidant activity should be measured by at least two independent procedures (8). The major biophenols in olives such as hydroxytyrosol can be found in the free form or linked to sugar and/or a secoiridoid moiety. Little is known about the effect of the structure on the bioactivity of olive biophenols and the possible synergy between these phenols.

In this study, the antioxidant and antiproliferative activities of olive biophenols plus crude and ethyl acetate extracts of olive mill waste (OMW)¹ were examined by a group of bioassays starting from the widely used in vitro model system involving

2,2'-diphenyl-1-picrylhydrazyl radical (DPPH[•]) scavenging to the various steps of oxidative damage (scavenging of superoxide radical SOR, scavenging of H₂O₂, and protecting DNA from HO[•]). The antiproliferative activities of test compounds were also investigated on cancer cell lines. Verbascoside, the most potent antioxidant identified in Australian OMW (9), and its constituent moieties, caffeic acid and hydroxytyrosol, were studied individually and in equimolar mixtures. Compounds previously recovered from OMW, namely, hydroxytyrosol acyclodihydroelenolate (HT-ACDE), *p*-coumaroyl-6'-secologanacoside (comselogoside), and 3,4-dihydroxyphenylethyl alcohol-deacetoxyelenolic acid dialdehyde (3,4-DHPEA-EDA) (Figure 1) (9–11), were examined for their antioxidant and antiproliferative activities.²

Experimental Procedures

Chemical Reagents. Reagents from the following sources were used without further purification. Nitrotetrazolium blue chloride (NBT), potassium phosphate monobasic, phenol red (ACS reagent), reduced β -nicotinamide adenine dinucleotide disodium salt (NADH), phenazine methosulfate, horseradish peroxidase (HRP) (type IV), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Sydney, Australia). Ferrous sulfate ($FeSO_4$) and hydrogen peroxide (H_2O_2) were obtained from Biolab (Sydney, Australia). Plasmid DNA (pBR322) was a product of Fermentas (Germany); McCoy's 5A medium modified (1×) containing L-glutamine and Ham's media (F-12 nutrient mixture) containing (1×) L-glutamine were from Invitrogen (Melbourne, Australia); fetal bovine serum (FBS) and penicillin/streptomycin (PS), 5000 IU/mL and 5000 μ g/mL, were obtained from Thermo Electron (Melbourne, Australia).

Samples and Standards. Biophenolic standards were caffeic acid (Sigma, Australia), hydroxytyrosol (Cayman, United States), oleu-

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¹ Abbreviations: 3,4-DHPEA-EDA, 3,4-dihydroxyphenylethyl alcohol-deacetoxyelenolic acid dialdehyde; DPPH[•], 2,2'-diphenyl-1-picrylhydrazyl radical; HRP, horseradish peroxidase; HT-ACDE, hydroxytyrosol acyclodihydroelenolate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT, nitrotetrazolium blue chloride; OMW, olive mill waste; ROS, reactive oxygen species.

² Chemistry and Bioassays of Antioxidant Biophenols.

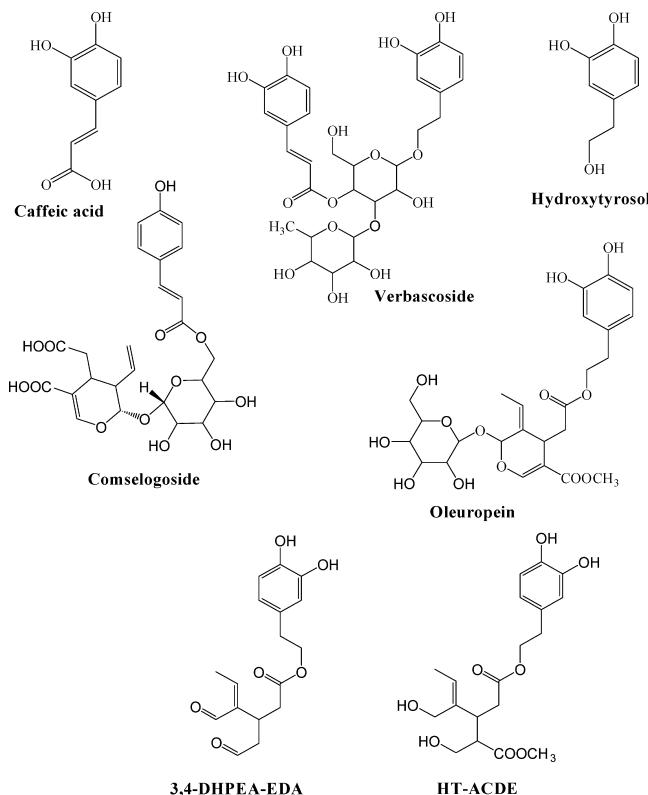


Figure 1. Structures of selected olive antioxidant biophenols.

ropein, and verbascoside (Extrasynthese, France). Purified compounds HT-ACDE, 3,4-DHPEA-EDA, and comsologoside were prepared as described previously (9, 10). Crude extract and ethyl acetate extract of Correggiola OMW collected in 2004 from Riverina Olive Grove were prepared as described in our previous work (9). The ethyl acetate solution was evaporated and reconstituted in 80% methanol. The methanol was evaporated under a stream of nitrogen, and the aqueous solution was freeze-dried overnight. All dried extracts and purified compounds were stored in a desiccator at -20°C .

Standards were prepared in 25% aqueous ethanol with the exception of caffeic acid and luteolin, which required 50% ethanol and absolute ethanol, respectively, for dissolution. The ethyl acetate extract was sparingly soluble in water; thus, initial dissolution in minimal volume of ethanol was necessary. All subsequent dilutions were made in the relevant phosphate buffer as mentioned below.

Scavenging of DPPH Radical. The assay was conducted as previously described (12).

Superoxide Radical Scavenging. The SOR scavenging activity was measured by the method of Robak and Gryglewski (13) with some modifications. The reaction was carried out in flat bottom 96 microplates. Fifty microliters of sample, 50 μL of NBT (200 μM), 50 μL of NADH (624 μM), and 50 μL of phenazine methosulfate (80 μM) were sequentially added. The reaction mixture was incubated at room temperature for 5 min, and the absorbance was read at 560 nm by Versamax Tunable (Molecular Devices, Sunnyvale, United States) automated microplate reader. Phosphate buffer solution was used as a negative control. Fresh phenazine methosulfate solution was prepared for every set of experiments as phenazine methosulfate solution in 0.1 M phosphate buffer (pH 7.4), although it was stored in amber-colored airtight screw cap containers, developed a blue coloration (pyocyanin) on standing. Phenazine methosulfate is known for its photosensitivity in aqueous solutions (14, 15). The % SOR scavenging activity was calculated according to the following equation:

$$\text{scavenging activity (\%)} = [1 - (A_{\text{sample}}/A_{\text{control}})] \times 100$$

Scavenging of Hydrogen Peroxide. A method described by Wang et al. (16) was used for the scavenging of H_2O_2 assay. All

of the reactions were carried out in flat bottom 96 microplates. Fifty microliters of freshly prepared 2 mM H_2O_2 solution was mixed with 50 μL of different concentrations of biophenols or OMW extracts prepared in 0.1 M phosphate buffer. The reaction mixture was incubated at room temperature ($20 \pm 2^{\circ}\text{C}$) for 20 min. The mixture (100 μL) made from freshly prepared HRP (0.3 mg/mL) and phenol red (4.5 mM) in 0.1 M phosphate buffer was added to the reaction mixture. After 10 min of incubation at room temperature, the absorbance was measured at 610 nm by an automated microplate reader. All samples and reagents were prepared in 0.1 M phosphate buffer (pH 7.4). Hydrogen peroxide solution was prepared freshly, and the concentration of hydrogen peroxide was determined by measuring the absorbance at 230 nm using $\epsilon_{\text{H}_2\text{O}_2, 230\text{ nm}} = 81 \text{ M}^{-1} \text{ cm}^{-1}$.

The % H_2O_2 scavenging activity was calculated according to the following equation:

$$\text{scavenging activity (\%)} = [1 - (A_{\text{sample}}/A_{\text{control}})] \times 100$$

Hydroxyl Radical-Induced Oxidative DNA Damage. Determination of the antioxidant (protective) and prooxidant (damaging) activity of OMW biophenols was conducted according to Tian and Hua with minor modifications (17). The reaction was conducted in polymerase chain reaction (PCR) microplates in a total volume of 15 μL . Plasmid DNA, pBR322, 0.5 $\mu\text{g}/\mu\text{L}$, was diluted 2-fold (0.5 $\mu\text{g}/3 \mu\text{L}$) using 50 mM phosphate buffer, pH 7.4. Diluted plasmid DNA (3 μL) was transferred to the microplate followed by 5 μL of the test substance at six different final concentrations for both individual biophenols and for the equimolar mixture of caffeic acid and hydroxytyrosol (1000, 500, 100, 50, 10, and 1 μM) as well as for extracts (0.1, 0.5, 1, 3, 4, and 5 mg/mL). The reaction mixtures were centrifuged for 30 s in a Sigma 4K15 centrifuge (Osterode, Germany) at 4°C . Iron(II) sulfate solution (2 mM; 3 μL) and 30% H_2O_2 (4 μL) were added successively and centrifuged for another 30 s. The reaction mixture was incubated in the dark at 37°C for 1 h, followed by addition of 3 μL of gel loading buffer [0.015% glycerol, 0.25% bromophenol blue, 0.13% cyanol blue, 10 mM Tris-HCl (pH 7.5), and 50 mM EDTA] and immediately stored at -83°C overnight. The mixture was allowed to thaw over ice and loaded on 0.9% agarose gel wells containing 250 mL of TBE buffer (0.89 M Tris base, 0.88 M boric acid, and 0.004 M EDTA) and 1 μL of ethidium bromide (10 mg/ml). Each reaction mixture was run horizontally in TBE buffer at 140 V for 2 h in an electrophoresis apparatus. The gels were photographed under UV light. For each run, a molecular marker (M), a negative control containing only DNA (D), hydrogen peroxide treatment (H), iron(II) treatment (F), and a positive control containing buffer instead of test sample (X) were loaded, as well as the various antioxidant treatments. The molecular marker used was Lambda HindIII (Promega, Australia). Band intensities were quantified by Quantity One software (BioRad, Australia), and average values from duplicate runs were presented.

Antiproliferative Activity. The antiproliferative activity of OMW extracts and biophenols was assessed using two adenocarcinoma cell lines, that is, colorectal adenocarcinoma (HT-29) and gastric adenocarcinoma (AGS). The cells were obtained from ATCC (American Type Culture Collection). HT-29 cells were cultured in McCoy's 5A medium, and AGS cells were cultured in Ham's medium, both containing 10% FBS and 1% PS at 37°C in 95% air with 5% CO_2 . Cells ($8-10 \times 10^3$ cells in 100 μL of medium) were plated into 96 well microplates and were incubated for 24 h. Subsequently, the cells were subjected to different concentrations of the test sample for 24 h. Ethanol was used to dissolve samples that were not phosphate-buffered saline (PBS) soluble. The final ethanol concentration did not exceed 2.5%. Controls were treated with relevant concentrations of ethanol. After 24 h, the plates were drained to remove all of the media and remnant samples and carefully washed with PBS. Fresh 100 μL of media was added followed by 10 μL of MTT (Sigma, Germany). The cells and MTT were incubated for 4 h. After 4 h, 100 μL of dimethyl sulfoxide (DMSO) was added to each well. The microplates were shaken in dark for 10 min to dissolve formazan crystals. The formazan product was determined by measuring the absorbance at 600 nm with a

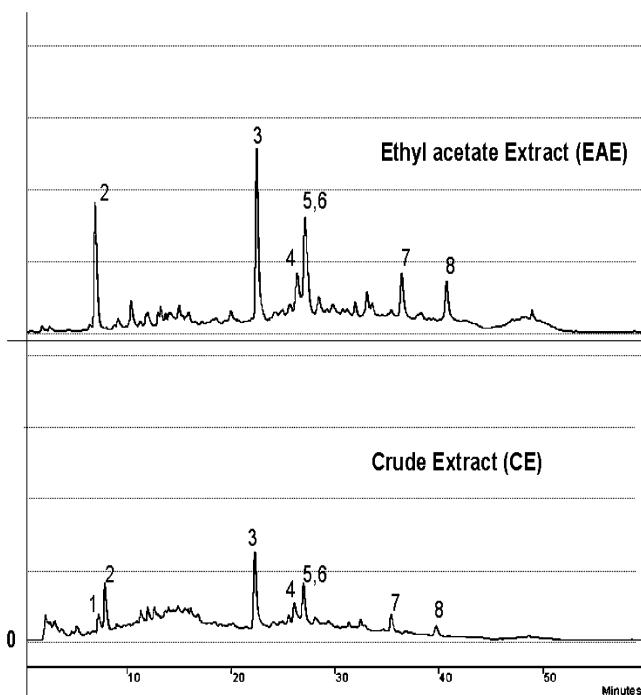


Figure 2. Chromatograms of crude extract and ethyl acetate extract on RP-HPLC at 280 nm showing the major peaks: 1, hydroxytyrosol glucoside; 2, hydroxytyrosol; 3, verbascoside; 4, rutin; 5, 3,4-DHPEA-EDA; 6, HT-ACDE; 7, comselogoside; and 8, luteolin.

Labsystem Multiscan RC (type 351) microplate reader (Helsinki, Finland) operated by the DeltaSoft3 program (Elisa Analysis for the Macintosh with interference for the Multiscan Microplate Readers, BioMetallics, Inc., 1995). HT-ACDE, 3,4-DHPEA-EDA, and comselogoside were not tested for their antiproliferative activity due to limited quantities available. The results were expressed as an optical density ratio of the control treatment. Student's *t* test was used to determine the difference between treatments and control at probability *p* < 0.05. Each concentration was tested in quadruplicate, and the whole experiment was repeated twice, giving eight readings for each concentration. The highest and the lowest readings were excluded, and the average \pm SD of *n* = 6 was used to present dose-response curves and calculate EC₅₀ with nonlinear regression.

Statistics. Analyses were performed in triplicate from at least two independent experiments. Results are presented as means \pm standard deviations. Outliers are tested and excluded by Grubbs' test (ISO recommendations) at *p* = 0.05. Data from bioassays were subjected to analysis of variance (ANOVA). Mean comparisons were performed using Duncan's test with SPSS software package v. Fourteen for Windows. EC₅₀ values were calculated as described earlier (12) using Microsoft Excel.

Results and Discussion

Extraction with ethyl acetate is widely used to enhance selectivity in the recovery of biophenols (18). Chromatograms at 280 nm of crude and ethyl acetate extracts of OMW from Correggiola are given in Figure 2, ethyl acetate extract representing the fraction of crude extract produced by removal of the highly polar nonphenolic and large molecular weight hydrophilic polymers (9, 19). However, the chromatograms also demonstrate that some phenolic compounds, for example, hydroxytyrosol glucoside, were also eliminated.

Antiradical Activity. The DPPH[•] antiradical activity (EC₅₀) of olive biophenols (Table 1) was in the following order: verbascoside \gg caffeic acid > HT-ACDE > oleuropein \approx hydroxytyrosol \approx 3,4-DHPEA-EDA > comselogoside. Differences to previously reported trends (20–22) may be attributed to methodology in terms of solvent choice (22). Comselogoside

had the lowest activity and was the only monophenol among the studied compounds. The small differences in activity of HT-ACDE, 3,4-DHPEA-EDA, and oleuropein reflect the importance of the phenolic hydroxyls in determining activity with minimal participation of the side chain substituents. The equimolar mixture of hydroxytyrosol and caffeic acid was more active than either compound individually but less active than verbascoside (Table 1). Experimentally, it was found that 7.80 μ M caffeic acid and hydroxytyrosol in a mixture resulted in 50% scavenging of DPPH[•] (Table 1); however, theoretically by substituting in nonlinear regression equations for caffeic acid [$y = 0.3654 \ln(x) - 0.3265$] and hydroxytyrosol [$y = 0.4449 \ln(x) - 0.7103$], 4.8 μ M caffeic acid and 7.60 μ M hydroxytyrosol are required to produce 50% scavenging of DPPH[•] assuming simple additive effects. While the activity of hydroxytyrosol was not significantly affected, a strong antagonistic effect of hydroxytyrosol on caffeic acid activity was noticed. This may involve some complex intermolecular interactions or subsidiary oxidation pathways. The esterification of the caffeic acid carboxyl and the spacing of the two pharmacophores from each other in verbascoside can not alone account for the high internal synergism in this molecule.

On the basis of DPPH[•] antiradical activity, ethyl acetate extract was 2.5-fold more active than the crude extract. This is consistent with the observation that nearly 30% of the weight of crude extract was highly polar matrix components with negligible antiradical activity and that was not extractable by ethyl acetate (9).

Superoxide Anion Radical Scavenging Activity. In the nonenzymatic phenazine methosulfate/NADH/O₂ system, phenazine methosulfate acts as an electron transfer catalyst between NADH and O₂, producing SOR (14) (eqs I and II, Figure 3). The nonscavenged SOR reduces the yellow, water-soluble nitroblue tetrazolium (NBT²⁺) radicals to blue, water-insoluble diformazan (DF) (eqs III–V, Figure 3). In aqueous solution and at physiological pH, phenols or their phenoxide ions can reduce SOR forming H₂O₂ (eqs VI and VII, Figure 3) (23). Thus, a more active SOR scavenger is indicated by a high absorbance reading for the yellow NBT²⁺. The biophenols were ranked according to their SOR scavenging activity (Table 1) into three groups: "100 μ M group" comselogoside \approx 3,4-DHPEA-EDA \approx verbascoside $>$ "300 μ M group" HT-ACDE \approx oleuropein $>$ "800 μ M group" caffeic acid \approx hydroxytyrosol.

The observed activity ranking suggests a major role of the nonphenolic component in determining the SOR scavenging activity. Verbascoside showed higher activity than hydroxytyrosol and caffeic acid alone or in equimolar mixture. Although the equimolar mixture of caffeic acid and hydroxytyrosol showed enhanced activity as compared with either of them alone, this was largely a simple additive effect. On the other hand, the combination of caffeic acid and hydroxytyrosol into the verbascoside molecule showed a synergistic effect. Both crude extract and ethyl acetate extract had good activity in the SOR scavenging assay comparable to that of pure compounds on a weight basis.

The chemistry of this test is complicated by a number of factors (24–29). For instance, compounds that are able to reduce NBT²⁺ will interfere (24). However, none of the tested phenols or OMW extracts had significantly reduced NBT²⁺ under the reaction conditions after 60 min (double the time required to complete the assay). Phenols can react with the H₂O₂ formed in eq VII, thereby reducing the extent of SOR scavenging that will subsequently be more available to react with NBT²⁺. However, comselogoside was the most active SOR scavenger

Table 1. Antioxidant Activities of OMW Biophenols and Extracts^a

biophenol(s) or extract	antioxidant activity (EC_{50})					
	DPPH [•] scavenging activity		SOR scavenging activity		H_2O_2 scavenging activity	
	ppm (μ g/mL)	μ M	ppm (μ g/mL)	μ M	ppm (μ g/mL)	μ M
hydroxytyrosol	2.34 ± 0.12	15.2 ± 0.78 a	124.8 ± 8.6	810 ± 56 a	70.4 ± 4.9	457 ± 31 a
caffeic acid	1.73 ± 0.05	9.6 ± 0.3 b	139.1 ± 8.6	772 ± 48 a	89.8 ± 4.8	498 ± 27 ab
oleuropein	7.58 ± 0.38	14.0 ± 0.7 a	213.6 ± 27.2	396 ± 50 b	312.1 ± 2.3	577 ± 4 b
verbascoside	1.84 ± 0.15	2.9 ± 0.2 c	69.2 ± 5.8	111 ± 9 c	175.3 ± 7.4	280 ± 12 c
caffeic acid and hydroxytyrosol	1.41 ± 0.01	7.8 ± 0.1 d	55.5 ± 5.2	308 ± 29 b	63.5 ± 1.2	353 ± 6 d
HT-ACDE	4.81 ± 0.13	12.6 ± 0.3 e	117.3 ± 0.3	307 ± 1 b	297.6 ± 14.6	779 ± 38 e
comselogoside	13.01 ± 0.58	24.23 ± 1.1 f	57.1 ± 1.6	106 ± 3 c	456.5 ± 16.9	852 ± 32 e
3,4-DHPEA-EDA	4.99 ± 0.24	15.6 ± 0.7 a	34.5 ± 1.8	108 ± 6 c	167.7 ± 12.4	524 ± 39 ab
CE	38.50 ± 1.93	NA	194.9 ± 10.1	NA	1481.5 ± 63.6	NA
EAE	14.49 ± 0.28	NA	147.1 ± 3.2	NA	682.3 ± 7.1	NA

^a Results are represented as means ± standard deviations of six readings from two independent experiments; NA, not applicable; CE, crude extract; EAE, ethyl acetate extract. Different letters in the same column indicate significantly different results ($p > 0.05$).

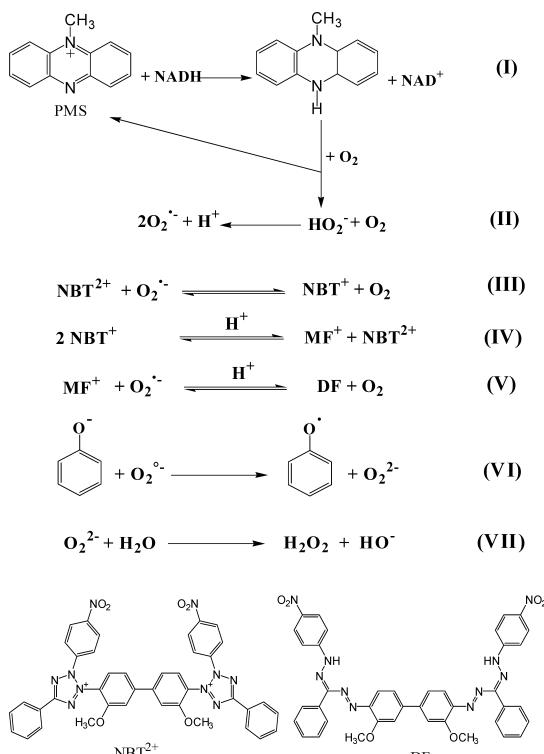


Figure 3. SOR production by phenazine methosulfate/NADH/O₂ system and reaction with NBT and phenols (33).

but the least active H_2O_2 scavenger (Table 1). At the same time, hydroxytyrosol and caffeoic acid, the least efficient scavengers of SOR had good H_2O_2 scavenging activity.

Hydrogen Peroxide Scavenging Activity. The peroxidase-based system (30) was used for measurement of hydrogen peroxide scavenging activity as direct measurement at 230 nm was unsuccessful due to very high interfering absorbance. In this system, the product of the HRP hydrogen peroxide-mediated oxidation of phenol red was measured at 610 nm, and the order of decreasing H_2O_2 scavenging activity was verbascoside > hydroxytyrosol ≈ caffeoic acid ≈ 3,4-DHPEA-EDA ≥ oleuropein > HT-ACDE ≈ comselogoside (Table 1). This ranking was very similar to that of DPPH[•] scavenging with the exception of the remarkably low activity of HT-ACDE in the H_2O_2 scavenging assay. Excluding verbascoside, the relative activities in SOR and H_2O_2 scavenging assays were very different. Esterification of hydroxytyrosol to secoiridoid residues generally reduced activity as compared to the free hydroxytyrosol. Among hydroxytyrosol secoiridoid derivatives, the dialdehyde (3,4-

DHPEA-EDA) was more active than the sugar residue (oleuropein), while HT-ACDE devoid of both came last. In contrast to the SOR scavenging activity, H_2O_2 scavenging activity mainly resulted from the phenolic moiety. Verbascoside showed an additive effect between its components, caffeoic acid and hydroxytyrosol, unlike their equimolar mixture, which had an antagonistic effect (Table 1). The ethyl acetate extract possessed more than double the activity of crude extract similar to DPPH[•] scavenging, which again reflects the difference in phenol content between the two extracts. Although the peroxidase method is the most widely used measure of hydrogen peroxide scavenging, different mechanisms including direct scavenging of H_2O_2 , inhibition of HRPO binding to H_2O_2 , and reaction with reaction intermediates (24) can contribute to the observed inhibition.

Protection of DNA against Damage by Hydroxyl Radical. Hydroxyl radical scavenging activity can be measured by the “deoxyribose assay” (30), but we preferred to measure the ability of antioxidants to protect DNA from HO^{\bullet} as this technique more closely simulates the *in vivo* situation. None of these biophenols have been tested previously for their DNA protection using plasmid DNA with the exception of verbascoside (31). However, the previous study used much higher concentrations of verbascoside (5 mM) but milder degradation conditions (no double-stranded DNA cleavage). As seen in Figure 4, hydrogen peroxide alone did not significantly affect supercoiled plasmid DNA (lane H), whereas Fe(II) exhibited single strand break leading to the formation of open circular plasmid DNA (lane F). A single-stranded nick on supercoiled plasmid DNA caused it to migrate as a compact band with relatively slower mobility (lanes H and F). As expected, H_2O_2 in combination with Fe(II) created double-stranded cleavage of plasmid DNA and led to generation of small fragments, degraded to a uniform size, which traveled to the bottom of the gel with little spread (lane X). Fenton reaction mediated DNA cleavage of double-stranded supercoiled plasmid DNA to produce a range of DNA fragments of varying sizes has not been described previously (17, 31).

Radical scavenging activity (DNA protection) resulted in migration behavior (lanes 1–6) that was between the two extremes. All compounds demonstrated protection against DNA damage relative to control X. The increase in the length of the smear can be attributed to the presence of larger fragments, signaling higher protection levels as seen in hydroxytyrosol, caffeoic acid, hydroxytyrosol and caffeoic acid equimolar mixture, HT-ACDE, comselogoside, 3,4-DHPEA-EDA, crude extract, and ethyl acetate extract (Figure 4; panels 1, 3, 4, and 5). From Figure 4, the biophenols can be ranked according to their ability

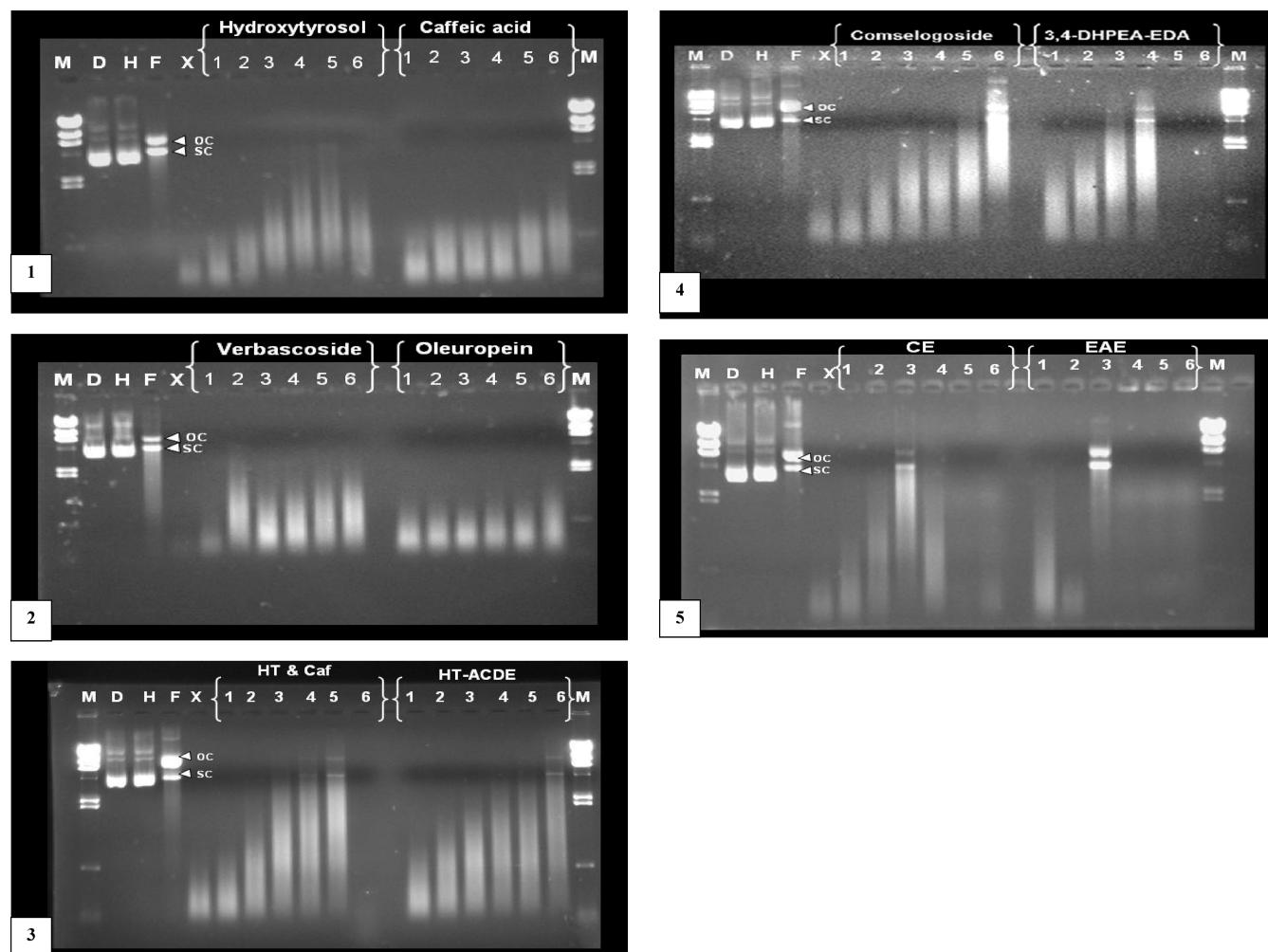


Figure 4. Agarose gel electropherogram showing the effect of various treatments on protection of DNA damage by hydroxyl radical. M, marker; D, untreated DNA (negative control); H, DNA treated with hydrogen peroxide only; F, DNA treated with Fe(II) only; X, DNA treated with hydrogen peroxide + Fe(II) (positive control); lanes 1–6 are DNA treated with hydrogen peroxide + Fe(II) with various concentrations of biophenols and extracts. Panels 1–4: lane 1, 1 μ M; lane 2, 10 μ M; lane 3, 50 μ M; lane 4, 100 μ M; lane 5, 500 μ M; and lane 6, 1000 μ M. Panel 5: lane 1, 0.1 mg/mL; lane 2, 0.5 mg/mL; lane 3, 1 mg/mL; lane 4, 3 mg/mL; lane 5, 4 mg/mL; and lane 6, 5 mg/mL. SC, “Form I” supercoiled plasmid DNA; OC, “Form II” open circular plasmid DNA.

to protect DNA from HO[•] damage: comselogoside > 3,4-DHPEA-EDA > hydroxytyrosol and caffeic acid equimolar mixture > HT-ACDE > hydroxytyrosol > verbascoside > caffeic acid > oleuropein. In contrast to the other assays, the equimolar mixture of caffeic acid and hydroxytyrosol was more active than verbascoside. However, verbascoside did not show any prooxidant activity up to 1 mM. OMW extracts showed high protection capacity of DNA damage at concentrations \leq 1 mg/mL. Interestingly, the monophenol comselogoside had the highest ability to protect DNA without showing any prooxidant activity in the tested range.

HT-ACDE, comselogoside, 3,4-DHPEA-EDA, and the equimolar mixture of caffeic acid and hydroxytyrosol showed bands for supercoiled plasmid DNA and open circular plasmid DNA (panels 3 and 4, Figure 4). Hydroxytyrosol and caffeic acid equimolar mixture and HT-ACDE had around 5% protection of supercoiled plasmid DNA at 500 and 1000 μ M, respectively. Comselogoside had the highest protection of supercoiled plasmid DNA (75%) of all tested samples at 1000 μ M, while 3,4-DHPEA had a maximum protection of supercoiled plasmid DNA (20%) at 100 μ M. Crude extract showed 17% protection of supercoiled plasmid DNA at 1 mg/mL, while at the same concentration, ethyl acetate extract showed 31% protection (panels 4 and 5, Figure 4). A prooxidant effect (enhancement

of DNA damage) was noticed for hydroxytyrosol and for hydroxytyrosol and caffeic acid equimolar mixture at 1000 μ M. 3,4-DHPEA-EDA had a prooxidant effect at 500 and 1000 μ M. OMW extracts showed good protection of DNA damage at lower concentrations (\leq 1 mg/mL), and prooxidant activity at concentrations \geq 3 mg/mL.

With the exception of verbascoside and oleuropein, all compounds demonstrated dose-dependent ability to inhibit HO[•] damage through progressive accumulation of high molecular weight supercoiled plasmid and open circular plasmid forms. Accordingly, verbascoside and oleuropein are proposed to have a different mechanism of action.

Antiproliferative Activity. Although there are a number of problems associated with the use of cell lines for testing antiproliferative effects, it does provide a viable option to the simplicity of in vitro model systems and to the use of animals and human subjects. Digestive system tumor cell lines were examined where the biophenols are expected to come in direct contact with the tumor cells at higher concentrations and where the effect of metabolism is minimal. Luteolin with an EC₅₀ = 146.0 \pm 13.1 μ M on HT-29 cells and EC₅₀ = 112.7 \pm 7.9 μ M on AGS cells was used as a positive control (Figures 5 and 6). The value for HT-29 cells is higher than previously reported EC₅₀ of 10.5 μ M (32). None of the tested biophenols exhibited

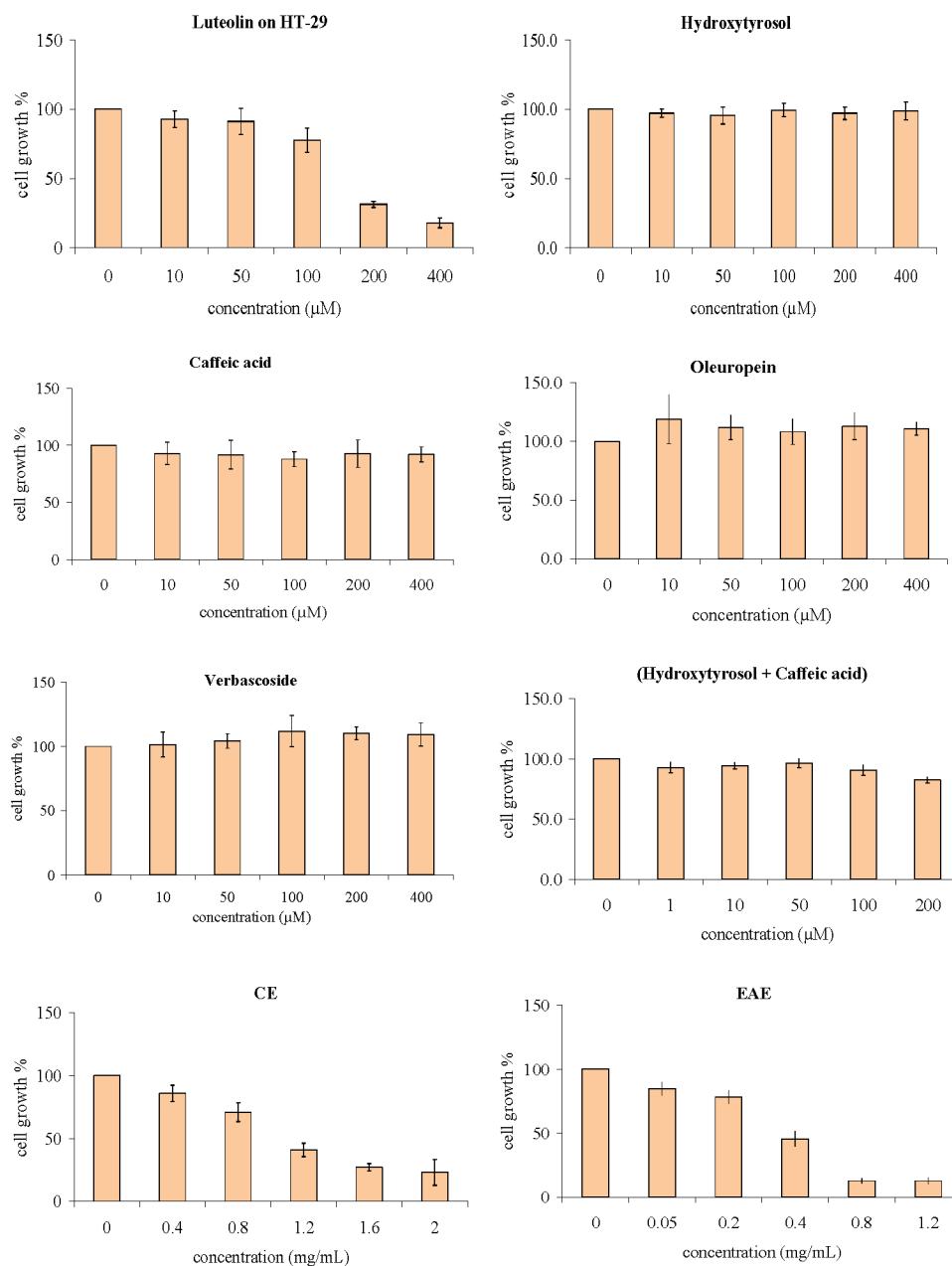


Figure 5. Effect of OMW biophenols and extracts on the growth of human colon cancer (HT-29) cells. Values are means \pm SD, $n = 6$.

a concentration-dependent decrease in the activity with an increase in concentration on both cell lines (Figure 5 and 6). In fact, none of them significantly affected the growth of HT-29 cells (Figure 5), and caffeic acid significantly promoted the growth of AGS cells at all applied concentrations. Hydroxytyrosol, oleuropein, and verbascoside at 10 μM significantly reduced the proliferation of AGS cells by 27, 16, and 19%, respectively. At higher concentrations of hydroxytyrosol and verbascoside, there was no significant change in antiproliferative activity; yet, at 400 μM , oleuropein significantly increased the proliferation of AGS cells (Figure 6).

The equimolar mixture of hydroxytyrosol and caffeic acid showed an antiproliferative effect on AGS cells that increased in the range 10–200 μM ($\text{EC}_{50} = 106.0 \pm 15.9 \mu\text{M}$). Although neither hydroxytyrosol nor caffeic acid significantly decreased the growth of HT-29 cells individually, their equimolar solution produced a small (ca. 5%) but significant decrease in proliferation at concentrations less than 50 μM ; a stepwise shallow

decrease pattern was observed at 50, 100, and 200 μM that was statistically significant at $p = 0.01$.

OMW extracts effectively inhibited the growth of both cell lines in a concentration-dependent manner (0.4–1.2 mg/mL). Ethyl acetate extract was more potent, and the AGS cell line was more sensitive than HT-29 (Figures 5 and 6). Ethyl acetate extract had an $\text{EC}_{50} = 0.289 \pm 0.017 \text{ mg/mL}$ on HT-29 cells and $\text{EC}_{50} = 0.248 \pm 0.027 \text{ mg/mL}$ on AGS cells. Crude extract had an $\text{EC}_{50} = 1.031 \pm 0.144 \text{ mg/mL}$ on HT-29 and $\text{EC}_{50} = 0.622 \pm 0.075 \text{ mg/mL}$ on AGS cells. At higher concentrations, both crude extract and ethyl acetate extract enhanced the growth of both cell types (data not shown). The observed low activity of individual biophenols can be due to a real resistance of the cells to their mechanism of action, lack of antiproliferative activity of these biophenols, or the degradation of the biophenols in the assay media. In the contrary, both crude extract and ethyl acetate extract effectively suppressed the growth of human cancer cells.

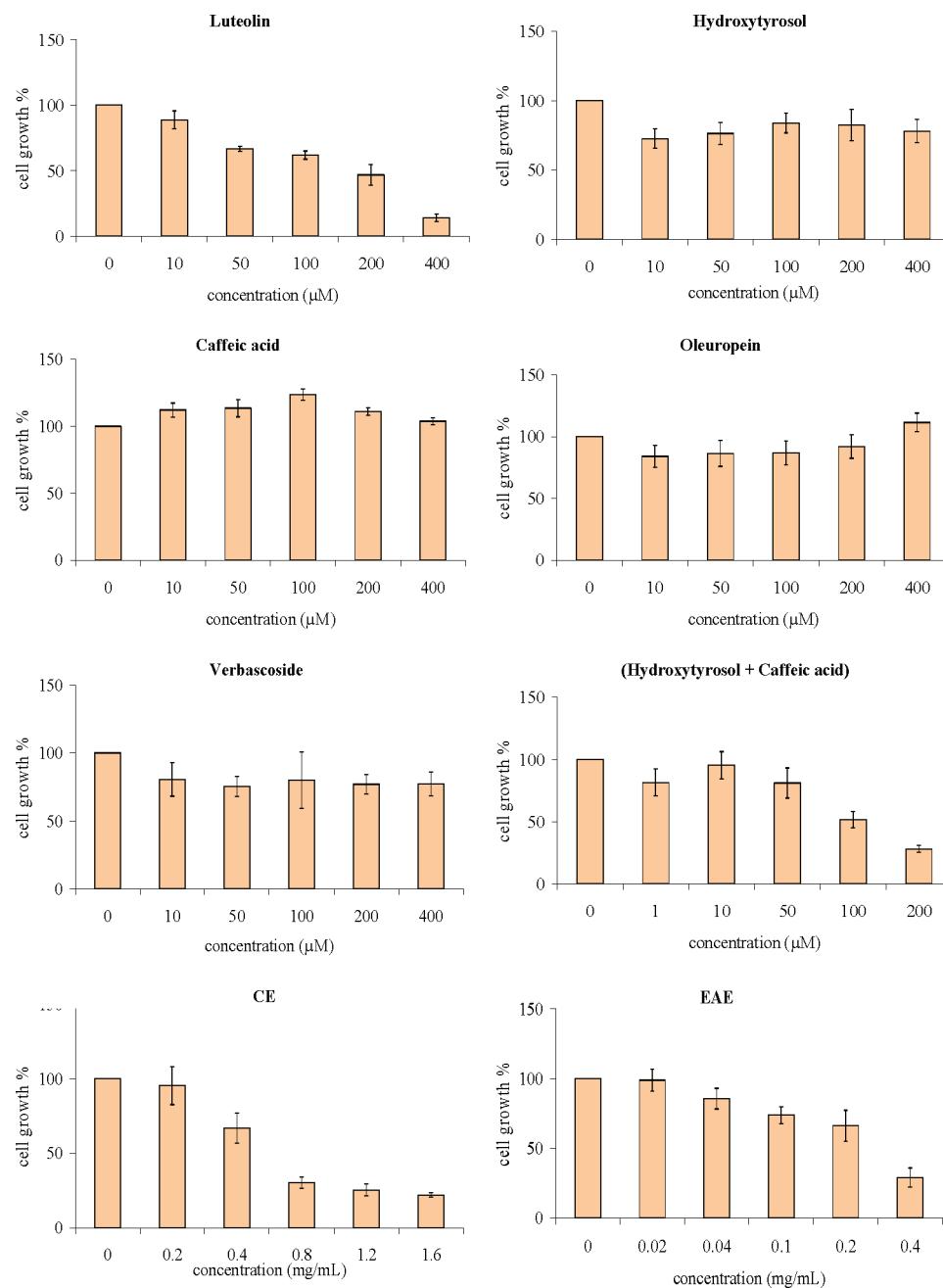


Figure 6. Effect of OMW biophenols and extracts on the growth of human stomach cancer (AGS) cells. Values are means \pm SD, $n = 6$.

Conclusion

The current study demonstrated antioxidant and antiproliferative effects of olive biophenols and extracts in a panel of assays. Individual biophenols were more active in the DPPH[•], SOR, and H₂O₂ scavenging assays, while extracts were more potent protectors of DNA from oxidative damage by Fenton reaction and as antiproliferative agents. Verbascoside was the most potent SOR and H₂O₂ scavenging compound. It protected DNA from oxidative damage in a dose-independent manner but had little antiproliferative activity on the AGS cell line, and it was inactive against HT-29 cell line. The novel secoiridoid isolated from Australian OMW, HT-ACDE, showed promising activity as a scavenger of reactive oxygen species (ROS) and as a chemopreventive agent protecting DNA from oxidative damage. The recently identified monophenol, comselogoside, had the highest activity as SOR scavenger and HO radical scavenger in phenazine methosulfate assay and DNA protection assay, respectively, although it was the least active biophenol

in DPPH and hydrogen peroxide assays. Biophenol extracts inhibited the proliferation of both HT-29 and AGS cell lines in a dose-dependent manner. Ethyl acetate extract was more potent as an antiproliferative agent on both cell lines than crude extract. Individual phenolic compounds had little or no activity on both cell lines. The equimolar mixture of hydroxytyrosol and caffeic acid dose dependently inhibited AGS cell line at concentrations $\geq 10 \mu\text{M}$. Biophenol extracts of OMW were effective in all applied assays with excellent antiproliferative activity.

The activities detected in such a diverse array of bioassays stress the putative role of olive biophenols as multiefficient biomolecules. The prooxidant activity noticed for some biophenols or OMW extracts was only observed at high concentrations that are probably not achievable under physiological conditions.

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