JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparison of Radical Scavenging Effect, Inhibition of Microsomal Oxygen Free Radical Generation, and Serum Lipoprotein Oxidation of Several Natural Antioxidants

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Typical components of the Mediterranean diet, such as olive oil and red wine, contain high concentrations of complex phenols, which have been suggested to have an important antioxidant role. The aim of the present work was to determine the inhibitory potency of compounds such as oleuropein, hydroxytyrosol, and other structurally related compounds, such as gallic acid, toward reactive oxygen species generation and free radical scavenging ability. The potency of these compounds was also examined with respect to protecting in vitro low-density lipoprotein oxidation. These studies indicate that complex phenols, such as hydroxytyrosol, and gallic acid both inhibit free radical generation and act as free radical scavengers. The use of three different approaches to determine antioxidant potency demonstrates that activity in one test does not necessarily correlate with activity in another. It was also demonstrated that the presence of two phenolic groups is not always associated with antioxidant activity.

KEYWORDS: Hydroxytyrosol; oleuropein; gallic acid; CYP3A; low-density lipoprotein; 2′,7′-dichlorofluorescin diacetate; 2,2-diphenyl-1-picrylhydrazyl radical

INTRODUCTION

Complex phenols, widely distributed in vegetables, are found in high concentrations in the typical components of the Mediterranean diet. The beneficial health effects of Mediterranean diets have been attributed, at least in part, to the presence of non-nutrient chemicals, including complex phenols such as hydroxytyrosol and oleuropein found in olive products. The quantities of these "minor components" or "non-nutrients" in olive oil vary, depending on a number of factors including the means of oil production and storage (*1*). The "non-nutrients" of olive oil have been suggested to have an important antioxidant role. For example, the olive oil minor components hydroxytyrosol and oleuropein have been shown to scavenge free radicals (*2*) and inhibit the chemical oxidation of low-density lipoproteins (*3*).

The structurally related compound gallic acid [a complex phenol found in red wine (*4*) and in both green and black teas (*5*, *6*)] has also been reported to scavenge free radicals (*7*, *8*), potentially contributing to the purported beneficial effects of tea (*9*).

However, a review of the published literature has indicated that although there has been much interest in the antioxidant potency of food-derived phenols, no single study has examined in a systematic manner the structure−activity relationships of these complex phenols.

Hepatic microsomal cytochromes P450 (CYP) are important in the metabolism of xenobiotic substrates, such as drugs, and endogenous substrates. The CYPs are also potentially a significant source of reactive oxygen species. Both superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) can be formed during the metabolism of compounds by CYPs (*10*, *11*). We have previously reported that the olive oil components oleuropein and hydroxytyrosol and the tea component gallic acid inhibited androstenedione 6β-hydroxylase activity, a CYP3A marker in human liver microsomes (*12*), potentially inhibiting reactive oxygen species generation.

The first goal of this study was to determine the inhibitory potency of a range of food-derived and related compounds (**Figure 1**, the components found in olive oil are highlighted by an asterisk) toward reactive oxygen species generation and free radical scavenging ability. The potency of these compounds was also examined with respect to protecting in vitro low-density lipoprotein oxidation.

The generation of reactive oxygen species was assayed by use of a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCF-DA), which has been used for the measurement of free radical generation (*10*, *11*). Free radical scavenging ability was determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging test widely used to determine free radical scavenging ability (*2*, *13*). The susceptibility of lipoproteins to oxidation was determined by monitoring lipoprotein diene formation directly in serum exposed to Cu^{2+} in vitro.

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10.1021/jf0112320 CCC: $22.00 © 2002 American Chemical Society
Published on Web 03/16/2002

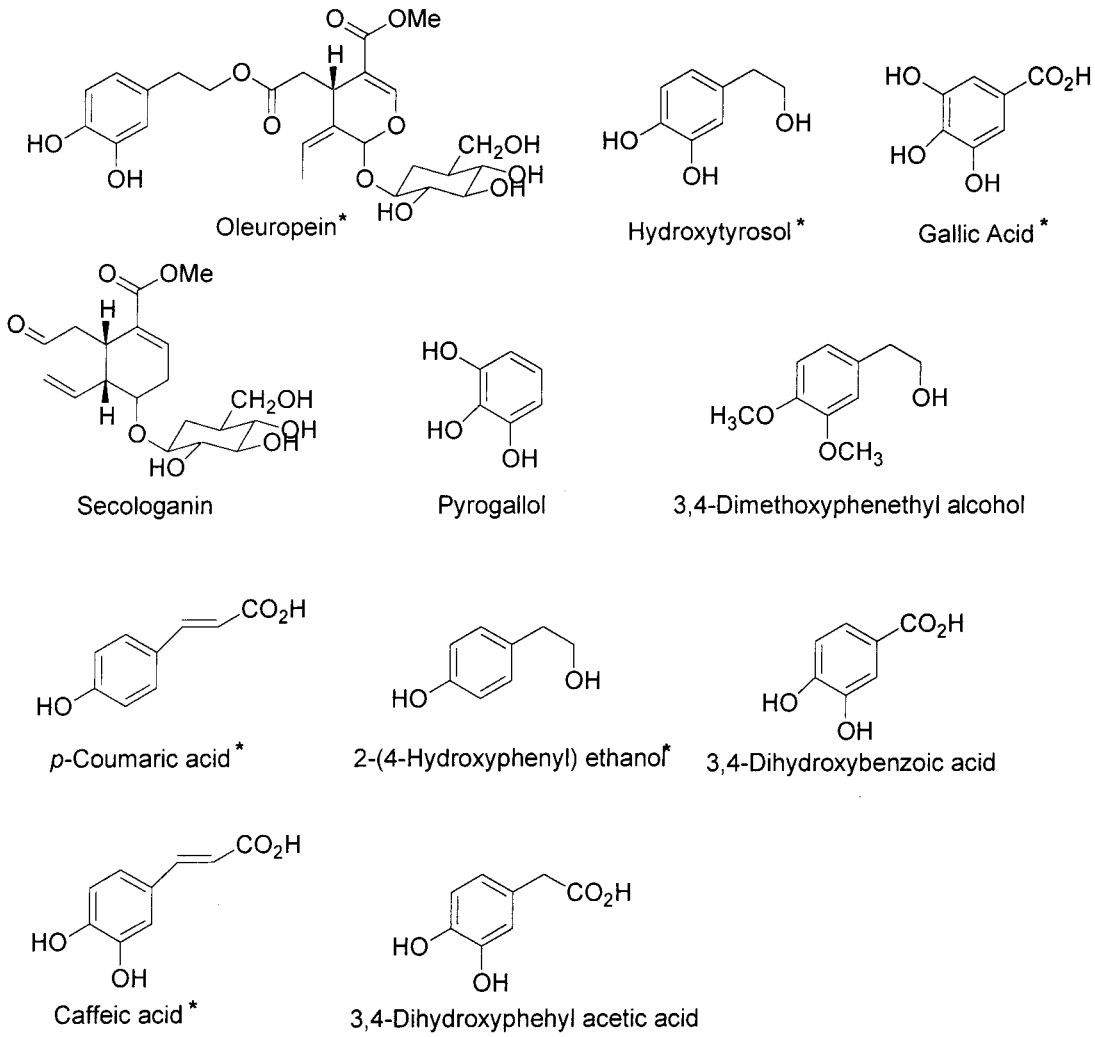

Figure 1. Structures of complex phenol compounds and other test compounds used in this study.

MATERIALS AND METHODS

Chemicals. NADP, NADPH, isocitrate dehydrogenase, pyrogallol, superoxide dismutase, and unlabeled androst-4-ene-3,17-dione (androstenedione) were purchased from the Sigma Chemical Co. (St. Louis, MO). [4-^{14}C]Androstenedione (specific activity = 2.0 GBq/mmol) was purchased from Amersham (Sydney, Australia). Other hydroxylated androstenedione standards were obtained from the MRC steroid collection or Steraloids, Inc. (Wilton, NH). Oleuropein was purchased from Indofine Chemicals (Somerville, NJ). Hydroxytyrosol was synthesized by the $LiAlH_4$ reduction of 3,4-dihydroxyphenylacetic acid (*14*). Gallic acid and *p*-coumaric acid were purchased from ICN, Aurora, OH; caffeic acid and 3,4-dihydroxybenzoic acid were purchased from BDH, Poole, U.K.; 2-(4-hydroxyphenyl)ethanol was purchased from Fluka, Buchs, Switzerland; and 3,4-dimethoxyphenethyl alcohol, 3,4-dihydroxyphenylacetic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were purchased from Aldrich, Milwaukee, WI. 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was purchased from Molecular Probes, Inc. (Eugene,OR), and 2′,7′- dichlorofluorescein (DCF) was obtained from Polysciences, Inc. (Warrington, PA). Secologanin was purchased from Phytoconsult (Leiden, The Netherlands). All solvents and other miscellaneous chemicals were at least of analytical reagent grade.

Microsomes. A sample of pooled human liver microsomes was purchased from Human Biologics International (Scottsdale, AZ). Male Hooded Wistar rats were used as the source of rat liver tissue. Hepatic microsomes were prepared from these livers by homogenization and differential centrifugation as previously described (*15*). Protein concentrations were determined as previously described (*16*). Reduced carbon monoxide spectra were recorded as described (*17*).

Estimation of Reactive Oxygen Species Generation. DCF-DA readily undergoes hydrolysis after crossing cell membranes to the nonfluorescent 2′,7′-dichlorofluorescin (DCFH). DCFH is rapidly oxidized in the presence of reactive oxygen species to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) (*10*, *11*).

Hepatic microsomes (0.2 mg) were incubated with DCF-DA (50 μM) and test compounds or superoxide dismutase (200 units/mL) in Tris buffer (40 mM, pH 7.4) for 25 min at 37 °C. In some cases the tubes were gassed with carbon monoxide at the start of the incubation in order to test total CYP inhibition. At the end of this incubation period, NADPH (0.6 mM) was added and the sample was incubated at 37 °C for a further 15 min. The formation of fluorescent product was determined using a Perkin-Elmer MPF-3L fluorescence spectrophotometer (excitation wavelength = 488 nm, emission wavelength = 525 nm). Product formation was determined using a DCF standard.

Microsomal Androstenedione Hydroxylation Assay. Microsomal androstenedione hydroxylation was assayed essentially as previously described (*12*). Microsomal fractions were incubated in a 1 mL of reaction mixture containing isocitrate (4 mM), isocitrate dehydrogenase (0.4 IU), $MgCl_2$ (8 mM), 1 mM NADP, and androstenedione (43.75 μM, 4.8 MBq/mmol) for 10 min at 37 °C, after which the incubation mixtures were extracted twice with ethyl acetate. Application to, and development of, TLC plates was carried out as previously described (*16*). Zones corresponding to hydroxylated androstenedione and to testosterone standards were visualized under UV light and scraped into vials for scintillation spectrometry (ACS, Amersham).

DPPH Radical Scavenging Test. Test compounds were added to a 50% ethanolic solution of DPPH radical (100 μM). The reaction

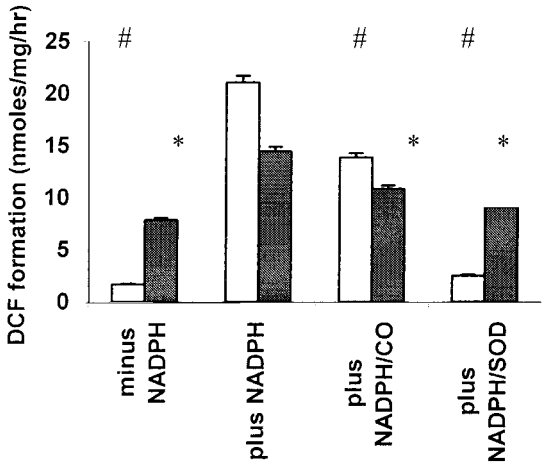

Figure 2. Hepatic microsomal reactive oxygen species generation: (unshaded bars) rat microsomal protein; (shaded bars) human microsomal protein; (#) significantly different from corresponding rat hepatic microsome control; (∗) significantly different from corresponding human hepatic microsome control ($P < 0.05$).

mixtures were then incubated with shaking at 25 °C. The absorbance of the remaining DPPH was determined at 517 nm using a UV-1601 Shimadzu spectrophometer. The scavenging activity was measured as the decrease in absorbance of the DPPH, expressed as a percentage of the absorbance of a control DPPH solution without test compounds.

Serum Oxidation. Analysis of lipoprotein diene formation was performed as described (*18*). Briefly, blood from six individuals was collected into glass, screw-cap tubes, left for 30 min in the dark at room temperature to clot, and then centrifuged for 20 min. The serum was collected, transferred to Eppendorf tubes, gassed with nitrogen, and then stored at −80 °C. To determine lag times, serum was diluted to a final concentration of 0.67% (v/v) in 0.02 M phosphate buffer, pH 7.4/0.16 M NaCl prewarmed to 30 °C. Freshly prepared $CuSO_4$ solution (50 μM final concentration) was added and the sample incubated at 30 °C for 180 min. Absorbance at 234 nm was determined every 15 min using a UV-1601 Shimadzu spectrophometer. In some experiments the test compounds were added at a range of concentrations. Lag time was measured from the plot of absorbance against time and was defined as the intercept between the baseline and the tangent of the absorbance during the propogation phase.

All assay conditions were optimized with respect to time, protein concentration, and substrate concentrations to ensure linearity.

Data Analysis. For each experiment, data are expressed as the means ± SD of three observations unless stated otherwise. Statistical analysis was carried out using one-way analysis of variance (STATVIEW, Berkeley, CA) followed by a Scheffe *F* test to detect differences.

RESULTS

Reactive Species Generation. To demonstrate the contribution of the CYP enzymes to microsome-mediated reactive oxygen species generation, DCF generation was measured in both human and rat liver microsomes. These experiments were conducted in the absence of our test antioxidant compounds. Results are presented in **Figure 2**. Reactive oxygen species generation was elevated by the inclusion of NADPH in the incubation mixtures ($P < 0.05$), although in human liver microsomes there was an ∼2-fold increase, whereas in rat liver microsomes the increase was ∼12-fold. The CYP content of both rat and human microsomes was determined to attempt to address this difference. The CYP contents of the human and rat liver microsomes were found to be 0.20 and 0.39 nmol/mg, respectively ($n = 2$). Gassing with carbon monoxide and inclusion of superoxide dismutase in the incubation mixture significantly decreased reactive oxygen species generation in microsomes from both rat and human liver ($P < 0.05$) (**Figure 2**).

Inhibition of Cytochrome P450 Activity. We have previously examined the inhibition of 6β-androstenedione hydroxylase activity (a CYP3A marker) in human liver microsomes by a range of olive oil phenols and related test compounds (*12*). The inhibition of both 6β- and 16α-androstenedione hydroxylase activities (CYP3A and CYP2C11 markers) in rat liver microsomes by these same compounds was also investigated. The compounds examined include the olive oil derived "nonnutrients" oleuropein and hydroxytyrosol and other structurally similar compounds. The results presented in **Table 1** demonstrate a range of inhibitory potencies with respect to inhibition of CYP3A in human liver microsomes and a range of inhibitory potencies with respect to inhibition of CYP3A and CYP2C11 in rat liver microsomes.

Inhibition of Reactive Species Generation. The data in **Table 1** also demonstrate a range of inhibitory potencies with respect to reactive oxygen species generation. The most potent inhibitors of reactive oxygen species generation in human liver microsomes were gallic acid > hydroxytyrosol > 3,4-dihydroxyphenylacetic acid. For rat liver microsomes the most potent inhibitors were hydroxytyrosol > 3,4-dihydroxyphenylacetic acid > oleuropein > pyrogallol > caffeic acid > gallic acid. Secologanin and 3,4-dimethoxyphenethyl alcohol, two structurally related compounds without phenolic hydroxyl groups, and three other tested compounds, *p*-coumaric acid, 2-(4-hydroxyphenyl)ethanol, and 3,4-dihydroxybenzoic acid, possessing one [*p*-coumaric acid and 2-(4-hydroxyphenyl)ethanol] or two phenolic hydroxyl groups (3,4-dihydroxybenzoic acid), were also essentially without inhibitory effect with respect to reactive oxygen species generation in both rat and human microsomal samples.

Correlation of human CYP3A activity and reactive oxygen species generation in the presence of test compounds gave $r^2 = 0.02$ (**Figure 3a**); a similar correlation with rat CYP3A and CYP2C11 gave correlations of $r^2 = 0.40$ and 0.41, respectively (**Figure 3**, panels b and c, respectively). Examination of the results obtained for pyrogallol and 3,4-dihydroxyphenylacetic acid indicate further features of the poor correlation obtained. Pyrogallol is an extremely potent CYP inhibitor (**Table 1**) (in both human and rat liver microsomes), yet in human it does not inhibit reactive oxygen species formation and in rat inhibits only 45% of reactive oxygen species formation. 3,4-Dihydroxyphenylacetic acid does not appear to be a potent inhibitor of CYP3A or CYP2C11 in rat liver microsomes, yet appears to inhibit 55% of the reactive oxygen species formation in rat liver microsomes (**Table 1**).

DPPH Radical Scavenging Test. The radical scavenging activity of the test compounds was also examined. Results are presented in **Table 2**. The most potent DPPH scavenging activity was apparent for gallic acid, pyrogallol, and 3,4-dihydroxyphenylacetic acid, then hydroxytyrosol, oleuropein, and caffeic acid. Those compounds without a phenolic hydroxyl group such as secologanin and 3,4-dimethoxyphenethyl alcohol were without effect for DPPH scavenging activity. 2-(4-Hydroxyphenyl)-ethanol and *p*-coumaric acid, which have only one phenolic group, also had no or negligible DPPH scavenging activity, respectively.

Serum Oxidation. Three concentrations (0.1, 1, and 10 μM) of those test compounds that inhibited reactive oxygen species generation and were determined to be reactive species scavengers were used to determine the changes in lag time for Cu^{2+}-

Table 1. Inhibition of Androstenedione Hydroxylase and Reactive Oxygen Species Generation in the Presence of Test Compounds

	androstenedione hydroxylase (nmol/mg/min)			nmol of DCF/mg/h	
test compound	human 6β-	rat 6β-	rat 16α-	human	rat
control	0.50 ± 0.05	0.66 ± 0.04	1.06 ± 0.12	14.4 ± 0.4	21 ± 0.7
oleuropein	0.30 ± 0.04[b,c]	0.51 ± 0.07	0.98 ± 0.09	16.2 ± 1.3	10.2 ± 1.2[b]
hydroxytyrosol	0.29 ± 0.05[b,c]	0.36 ± 0.06[b]	0.95 ± 0.08	11.4 ± 0.6[b]	8.4 ± 0.2[b]
gallic acid	0.25 ± 0.03[b,c]	0.43 ± 0.05[b]	0.60 ± 0.07[b]	10.2 ± 0.6[b]	14.4 ± 0.7[b]
secologanin	0.44 ± 0.11	0.74 ± 0.12	1.16 ± 0.11	14.4 ± 0.6	21.0 ± 0.3
pyrogallol	0.08 ± 0.02[c]	0.10 ± 0.05[b]	0.26 ± 0.05[b]	16.8 ± 0.5	11.4 ± 0.4[b]
3,4-dimethoxyphenethyl alcohol	0.43 ± 0.06[c]	0.53 ± 0.04	1.11 ± 0.13	13.2 ± 0.7	25.2 ± 0.6
p-coumaric acid	0.46 ± 0.05[c]	0.62 ± 0.04	1.11 ± 0.10	15.6 ± 0.5	24.0 ± 0.6
2-(4-hydroxyphenyl)ethanol	0.44 ± 0.05[c]	0.56 ± 0.05	1.02 ± 0.14	19.8 ± 0.7	21.6 ± 0.6
3,4-dihydroxybenzoic acid	0.40 ± 0.05[c]	0.56 ± 0.04	0.93 ± 0.15	15.0 ± 0.4	22.8 ± 0.7
caffeic acid	ND[d]	0.52 ± 0.06	1.06 ± 0.12	21.0 ± 1.2	13.8 ± 0.4[b]
3,4-dihydroxy phenylacetic acid	ND	0.47 ± 0.07	0.82 ± 0.09	12.0 ± 0.7	9.6 ± 0.4[b]

[a] Test compounds (100 μM) were included in both androstenedione hydroxylase assays and assays for reactive oxygen species generation (NADPH stimulated). [b] Statistically less than control i.e., without addition of test compound ($P < 0.05$). [c] Previously reported from this laboratory (*12*). [d] ND, not done.

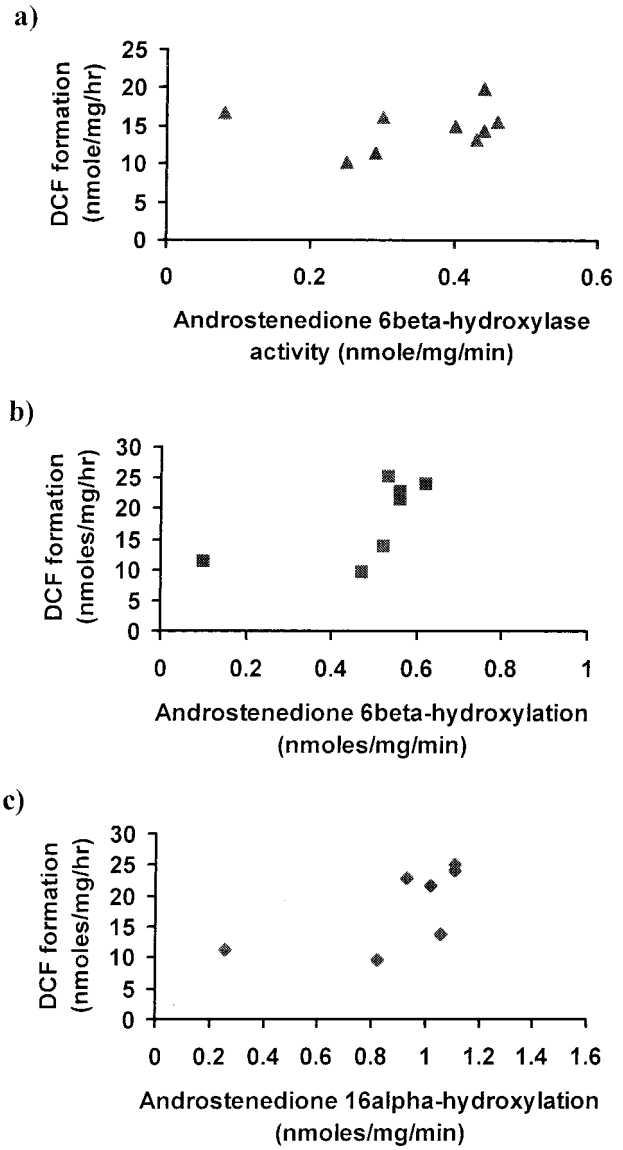

Figure 3. Correlation between inhibition of androstenedione hydroxylation and reactive oxygen species by complex phenols and related compounds: (a) human liver microsomes; (b, c) rat liver microsomes.

induced lipoprotein diene formation observed with serum from one individual. Results using 1 μM of three of the test compounds are shown in **Figure 4**. The data clearly indicate that oleuropein, hydroxytyrosol, and gallic acid delay lipoprotein diene formation. The most appropriate concentrations from these experiments were then used to determine changes in lag time observed with serum from six individuals. Results are presented in **Table 3**.The data show that there is a statistically significant increase in lag time with 1 μM oleuropein, hydroxytyrosol, and gallic acid and with 10 μM 2-(4-hydroxyphenyl)ethanol, whereas secologanin and 3,4-dimethoxyphenethyl alcohol have essentially no effect.

DISCUSSION

Antioxidants may be put into two separate groups: those that suppress the generation of reactive oxygen species and those that scavenge the reactive oxygen species generated. A review of the published literature indicated that no systematic study had been carried out which compared the inhibition of reactive species generation and reactive species scavenging.

We have utilized a rapid and sensitive assay to measure free radical generation in rat and human liver microsomes (*10*, *11*, *19*). Human CYP3A has been reported to have the highest capacity for reactive oxygen species generation on a per nanomole basis of all CYPs (*19*). It was therefore of interest to examine the effects of dietary phenols such as oleuropein and hydroxytyrosol, known CYP3A inhibitors (*12*), and other structurally related compounds on human hepatic reactive oxygen species generation. We compared inhibition of reactive oxygen species generation and inhibition of selected CYPs comparing rat (CYP2C11 and CYP3A) and human liver microsomes (CYP3A).

Initial experiments validated our methods for quantification of reactive oxygen species generation with DCF-DA (**Figure 2**). We demonstrated NADPH dependence and inhibition by carbon monoxide in microsomes from both species. Interestingly, human hepatic microsomes did not show the same extent of stimulation by addition of NADPH or the same extent of inhibition after bubbling with carbon monoxide. Reactive radical species generation has been demonstrated for CYPs; however, other drug-metabolizing enzymes such as NADPH-cytochrome P450 reductase (reviewed in ref *20*) may play a role. Other studies (*20*) have shown a rate of reactive oxygen species generation in human microsomes of 20−30% of that in rat liver microsomes, whereas the NADPH-stimulated rate reported in our study was on the order of 60% of the rate obtained in rat liver microsomes; however, as pointed out (*20*) the ratio of CYP, NADPH-cytochrome P450 reductase, and NADH-cytochrome *b*5 reductase may be relevant. Inhibition of NADPH-stimulated

Table 2. Scavenging Effects of Test Compounds on the DPPH Radical

test compound	10.0 μM	7.5 μM	5 μM	2.5 μM	1.25 μM
oleuropein	44 ± 3	32 ± 2	21 ± 3	9 ± 4	4 ± 3
hydroxytyrosol	47 ± 3	37 ± 4	22 ± 3	12 ± 3	5 ± 2
gallic acid	87 ± 5	87 ± 3	71 ± 2	36 ± 2	21 ± 2
secologanin	0	0	0	0	0
pyrogallol	72 ± 4	57 ± 2	39 ± 2	19 ± 3	10 ± 2
3,4-dimethoxyphenethyl alcohol	0	0	0	0	0
p-coumaric acid	3 ± 2	3 ± 3	2 ± 3	1 ± 3	0
2-(4-hydroxyphenyl)ethanol	0	0	0	0	0
3,4-dihydroxybenzoic acid	20 ± 2	14 ± 2	9 ± 2	6 ± 2	1 ± 2
caffeic acid	35 ± 3	30 ± 4	20 ± 3	9 ± 2	4 ± 1
3,4-dihydroxyphenylacetic acid	75 ± 4	57 ± 3	38 ± 4	23 ± 2	8 ± 1

[a] The results are expressed in % = [(*A* in the absence of compound − *A* in the presence of compound)/*A* in the absence of compound] × 100.

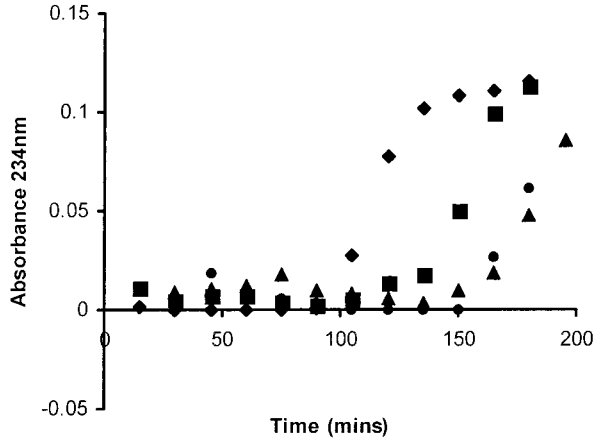

Figure 4. Lipoprotein diene formation: (◆) serum with addition of no compound; (●) serum with addition of 1 μM oleuropein; (▲) serum with addition of 1 μM gallic acid; (■) serum with addition of 1 μM hydroxytyrosol.

Table 3. Lag Time to Lipoprotein Diene Formation, $n = 6$ Individual Serum Samples

test compound and concn	increase in lag lag time relative to control (min)	*P* value
oleuropein (1 μM)	65 ± 12	<0.001
hydroxytyrosol (1 μM)	60 ± 13	<0.001
gallic acid (1 μM)	45 ± 19	<0.003
secologanin (10 μM)	none	not statistically different
3,4-dimethoxyphenethyl alcohol (10 μM)	none	not statistically different
p-coumaric acid (10 μM)	20 ± 8	<0.002
2-(4-hydroxyphenyl)-ethanol (10 μM)	30 ± 9	<0.001

reactive oxygen species formation by superoxide dismutase suggests that superoxide is an obligatory intermediate.

Levels of NADPH in rat liver are in the range of 200 nmol/g of hepatic tissue (*21*). Levels in human liver have not been reported but are expected to be similar. Thus, a significant amount of free radical generation may occur in hepatic cells. In vivo, in human liver tissue, food-derived hydroxytyrosol and gallic acid and the structurally related compound 3,4-dihydroxyphenylacetic acid would be expected to decrease reactive oxygen species generation.

Measurement of the scavenging effects of test compounds on the DPPH radical were generally in agreement with those in the literature where available. Results for gallic acid have been reported from two other laboratories (*7*, *8*); IC_{50} values of 28 and 11 μM, respectively, have been reported. In our study hydroxytyrosol and oleuropein were found to have similar DPPH scavenging capacities when one test compound was compared against the other. A previous study (*2*) reported values that indicated a scavenging capacity of hydroxytyrosol which was 2 orders of magnitude greater than that for oleuropein. From our results (**Table 2**) we can observe that only those compounds possessing two phenolic hydroxyl groups, in an ortho position, were able to act as DPPH radical scavengers. Our results are in agreement with the work of others (*22*), who have shown using techniques such as electron spin resonance and a thiobarbituric acid method, that, in general, catechol compounds possess antioxidant activity.

Our studies indicate that olive oil phenols, such as hydroxytyrosol, and other food-derived compounds, such as gallic acid, both inhibit free radical generation and act as free radical scavengers. Our study is the first systematic study comparing both parameters using individual, highly purified compounds. We have not investigated the possibility that with ingestion of a mixture of such compounds in foodstuffs synergistic or antagonistic effects may be observed. In recent years free radicals have been linked to the pathogenesis of several diseases such as atherosclerosis (reviewed in ref *23*). These findings provide additional mechanistic information for the beneficial effect of the olive oil phenolics such as oleuropein and tea-derived gallic acid.

Our studies, using the three different approaches to determine antioxidant potency, demonstrate that activity in one test does not necessarily correlate with activity in another. For example, *p*-coumaric acid and 2-(4-hydroxyphenyl)ethanol showed very little activity in the DPPH scavenging and DCF reactive oxygen species generation test; however, the increase in lag time in tests to determine lipoprotein diene formation was statistically significant. We have also demonstrated that the presence of two phenolic groups is not always associated with antioxidant activity, for example, 3,4-dihydroxybenzoic acid.

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Received for review September 21, 2001. Revised manuscript received January 29, 2002. Accepted January 29, 2002.

JF0112320