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Antioxidant Effect of Two Virgin Olive Oils Depends on the Concentration and Composition of Minor Polar Compounds

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In vitro studies show that some individual minor polar phenolic compounds (MPC) present in virgin olive oil prevent oxidation of human low-density lipoproteins (LDL), but few data are available on the antioxidant effect of whole oil extract. Thus, whole virgin olive extracts were studied to determine whether they maintain the antioxidant activity and whether this last is linked to MPC composition of a single virgin oil. Using HPLC-DAD the MPC content in Taggiasca and Seggianese virgin olive oils was measured. Taggiasca oil was less rich in total MPC (208.5 mg/L) than Seggianese oil (441.9 mg/L). In addition, the major compounds of Taggiasca oil were lignan derivatives, whereas the major compounds in Seggianese oils were secoiridoid derivatives. Moreover, Taggiasca oil was practically free of 5-hydroxytyrosol and 5-hydroxytyrosol derivatives, deacetoxy-oleuropein aglycone and oleuropein aglycone. The antioxidant activity of the oils on human LDL was evaluated by measuring malondialdehyde and conjugate diene generation induced by copper ions. In both tests, the oil extracts dose-dependently reduced malondialdehyde and conjugate diene generation. Moreover, antioxidant potency correlated with total MPC; thus, Seggianese extract was more active. The two oils differed quantitatively and qualitatively, and these differences influenced their biological activities; thus clinical trials focused on studying the effects of olive oils should specify the oils used.

KEYWORDS: Antioxidant activity; LDL; MDA; secoiridoids; lignans; deacetoxy-oleuropein; acetoxy-pinoresinol; virgin olive oil

INTRODUCTION

Epidemiological studies suggest that Mediterranean diets are associated with a reduced risk of cardiovascular diseases, the lower incidence of cardiovascular disease being associated with greater adherence to the Mediterranean diet (*1*−*3*). Recently, it has been shown in more than 3000 individuals, without clinical evidence of cardiovascular disease, that total plasma antioxidant capacity and low oxidized low-density lipoproteins (LDL) are associated with greater adherence to the Mediterranean diet (*4*). Compared with a saturated fat diet, the Mediterranean diet, rich in oleic acid, has also been associated with lower LDL and total triglycerides with maintenance of high-density lipoproteins (*5*−*7*) and lower blood pressure (*8*, *9*).

Olive oil composition depends on many factors such as olive cultivar (*10*−*12*) and agronomic and technological aspects of production (*13*−*15*). Virgin oil, which is obtained directly from pressing ripe olives, retains sizable amounts of minor polar compounds (MPC) and tochopherols (*16*), which can act as antioxidants (*17*). Recently, it was shown that the antioxidant activity of monovarietal extra virgin olive oils was increased in those oils extracted from destoned fruits and that this effect was variety-dependent (*18*).

It is known that some single MPC increase the resistance of LDL against oxidation in vitro (*19*−*23*), but the single MPC approach fails to account for the interactions among MPC and does not take into consideration that some MPC are correlated between them. In this respect, it is important to note that the mixture of phenols may exert different activity in comparison with the single phenols, because they may cooperate, thereby modifying biological activity (*24*). Interactions among phenols seems also to depend on the relative amount of single polyphenols (*25*). Thus, individual olive oils, which differ qualitatively and quantitatively, could have different biological activities. At present, little is known about the antioxidant effect of the total

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olive oil extract. In addition, the relationship between antioxidant activity of whole virgin olive oil and its global MPC content is not yet known (*26*, *27*). The foregoing observations may account for the conflicting results obtained in clinical trials designed to determine the effect of olive oil (*27*−*31*). Interestingly, in two of these studies, the beneficial effects of virgin olive oil were related to MPC amount (*27*, *30*). Olive oil is a basic component of the Mediterranean diet; thus, it is important to identify and quantify the antioxidant compounds in individual virgin oils so as to determine which have the healthiest effects.

MATERIALS AND METHODS

Tyrosol, luteolin, and oleuropein were obtained from Extrasynthèse (Genay, France). 5-Hydroxytyrosol was purchased from Cayman Chemical (SPI-BIO, Montigny le Bretonneux, France). Solvents for the high-performance liquid chromatography−diode array detection (HPLC-DAD) analyses were of analytical grade and were purchased from Carlo Erba (Milan, Italy). Other reagents were of analytical grade and were purchased from Sigma (St. Louis, MO).

Preparation, Characterization, and Quantification of Virgin Olive Oil Extracts. The virgin oils were produced from two autochthonous cultivars: 'Olivastra Seggianese' (Tuscany, Italy) and 'Taggiasca' (Liguria, Italy). The olives were processed immediately after harvesting by Manni S.p.A. (Grosseto, Italy) and Isnardi S.p.A. (Imperia, Italy), respectively. Sample preparation and extraction and identification, characterization, and quantification of single polar compounds were carried out as previously reported (*32*). Briefly, 50 mL of each fresh oil sample was extracted with 150 mL of ethanol and formic acid acidified water (70:30 v/v). Defatting with *n*-hexane was carried out to remove the lipid fraction. The extract was concentrated under reduced pressure to dryness, dissolved with 2 mL of extraction solvent, and analyzed by HPLC using an HP-1100 liquid chromatograph equipped with a DAD detector and an HP 1100 MSD API−electrospray (Agilent Technologies, Palo Alto, CA). The MPC were identified on the basis of their retention times and spectroscopic and spectrometric data, using 5-hydroxytyrosol, tyrosol, luteolin, and oloeuropein as reference compounds. Lignan was identified and analyzed as described in Mulinacci et al. (*33*). Oleocanthal was identified according to the method of Beauchamp et al. (*34*). The single minor compounds were quantified with HPLC-DAD using a four-point regression curve constructed with the available standards. Calibration curves with $r^2 \geq 0.9998$ were used (*11*). In all cases actual concentrations of derivatives were calculated after the application of corrections for changes in molecular weight: knowing the molecular weight of each compound (PM_x) allowed its actual concentration to be obtained by applying a multiplication factor of PM_x/PM_y, where PM_y is the molecular weight of the specific reference compound. The same extract analyzed by HPLC was used for the LDL test.

Subjects. Thirty healthy, sex-matched volunteers gave their informed consent to participate in the study. Smokers were excluded from the study. No subject had a family history of diabetes, hypertension, or dyslipidemia. They remained free of drugs, vitamins, amino acids, hormones, dietary supplements, and botanical remedies for the 14 days preceding the study. All of the subjects regularly consume a typical Mediterranean diet.

LDL Isolation and Preparation. In the morning after a 12-h fast, 40 mL of blood was collected by venipuncture into EDTA-containing vacutainer tubes (1 g/L) and centrifuged immediately for 10 min at 2000*g* at 4 °C. LDL was isolated from plasma with the discontinuous ultracentrifugation method using a TL-100 tabletop ultracentrifuge (Beckman, Palo Alto, CA) as reported previously (*35*) with minor revisions. To protect the LDL against oxidative alterations during ultracentrifugation, each density solution contained EDTA. The samples were then exhaustively dialyzed in PD-10 desalting columns (Amersham Pharmacia Biotech, Uppsala, Sweden) to remove excess salt and most of the EDTA. The LDL samples were used immediately thereafter.

Biochemical Assays. Total cholesterol, LDL, high-density lipoprotein (HDL), and triglycerides were measured as previously described (*36*). The protein concentration of LDL was determined using bovine serum albumin as standard (*37*). LDL was diluted to 50 μg of protein/mL, and LDL oxidation was carried out as previously described in Romani et al. (*25*). Oxidation was initiated by adding freshly prepared 5 μM $CuSO_4$ (final concentration). LDL oxidation was measured in triplicate with the thiobarbituric acid method, using malonaldehyde bisdiethylacetal as standard (*38*). The malondialdehyde was measured in basal condition and after 8 h of exposure to copper ions, in the presence and absence of different concentrations of extra virgin oil extracts added before copper ions. Conjugated diene formation was determined at a temperature of 37 °C and by monitoring the absorbance at 234 nm, as described elsewhere (*39*).

Statistical Analysis. The results are expressed as mean ± standard error (SE). IC_{50} was calculated using the Sigmaplot 8.0 program (SPSS Inc., Chicago, IL). ANOVA followed by Turkey's multiple-range test was used to compare means at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

As shown in **Figure 1** and **Table 1**, the MPC identified and quantified in the two virgin olive oils belong to three classes: simple phenols (tyrosol, 5-hydroxytyrosol, and 5-hydroxytyrosol derivatives), secoiridoids (oleuropein aglycones, deacetoxy-oleuropein aglycone, oleocanthal, and secoridoids derivatives), and lignan derivatives (acetoxypinoresinol). The Taggiasca extract contained a high percentage of lignan derivatives (72.5%) and no 5-hydroxytyrosol, 5-hydroxytyrosol derivatives, deacetoxy-oleuropein aglycone, or oleuropein aglycone (**Figure 1** and **Table 1**). The Seggianese extract contained tyrosol, 5-hydroxytyrosol, 5-hydroxytyrosol derivatives, deacetoxy-oleuropein aglycone, and oleuropein aglycone. In addition, it was rich in secoridoids and had a relatively low amount of lignan derivatives. Both oil extracts contain oleocanthal, the Saggianese cultivar being richer than the Taggiasca (**Figure 1** and **Table 1**). 5-Hydroxytyrosol and oleuropein aglycone show strong antioxidant properties (*40*). Oleocanthal is reported to be an inhibitor of cyclooxygenases 1 and 2 (*34*); on the other hand, there are no studies about antioxidant properties of deacetoxy-oleuropein aglycone as a single compound. Acetoxypinoresinol shows only low antioxidant properties (*41*), and to our knowledge tyrosol and elenolic acid do not possess any antioxidant properties (*42*).

None of the extracts affected the basal level of malondialdehyde (data not shown) in LDL from healthy individuals. Hence, we evaluated the effect of the oil extracts on malondialdehyde production induced by copper ions in human LDL. We found that the olive oils dose-dependently inhibited malondialdehyde generation, and the IC_{50} showed that the Seggianese extract was the most potent when total MPC were considered. The shapes of dose−response curves differed, but when efficiency was calculated, the difference was not significant (data not shown). Measurable LDL conjugated diene formation did not occur in the absence of copper ions (data not shown). Oil extracts shifted the conjugated diene formation curve to the right (**Figure 2**).

Diet is a cornerstone of cardiovascular disease prevention (*43*), and epidemiological studies demonstrate that Mediterranean populations have a lower incidence of cardiovascular disease (*44*). The healthy effect of the Mediterranean diet has been attributed to the consumption of large amounts of fiber, fruits, vegetables, and unsaturated fatty acid provided by olive oil. However, the contribution of virgin olive oil as a major source of dietary antioxidants has not been investigated in depth (*45*). Our data confirm that virgin olive oils contain antioxidants in abundance (*11*, *32*). The level of MPC was about 2.1 higher in Saggianese oil extract than in the Taggiasca one, which indicates that the cultivar used to produce the oil is an important

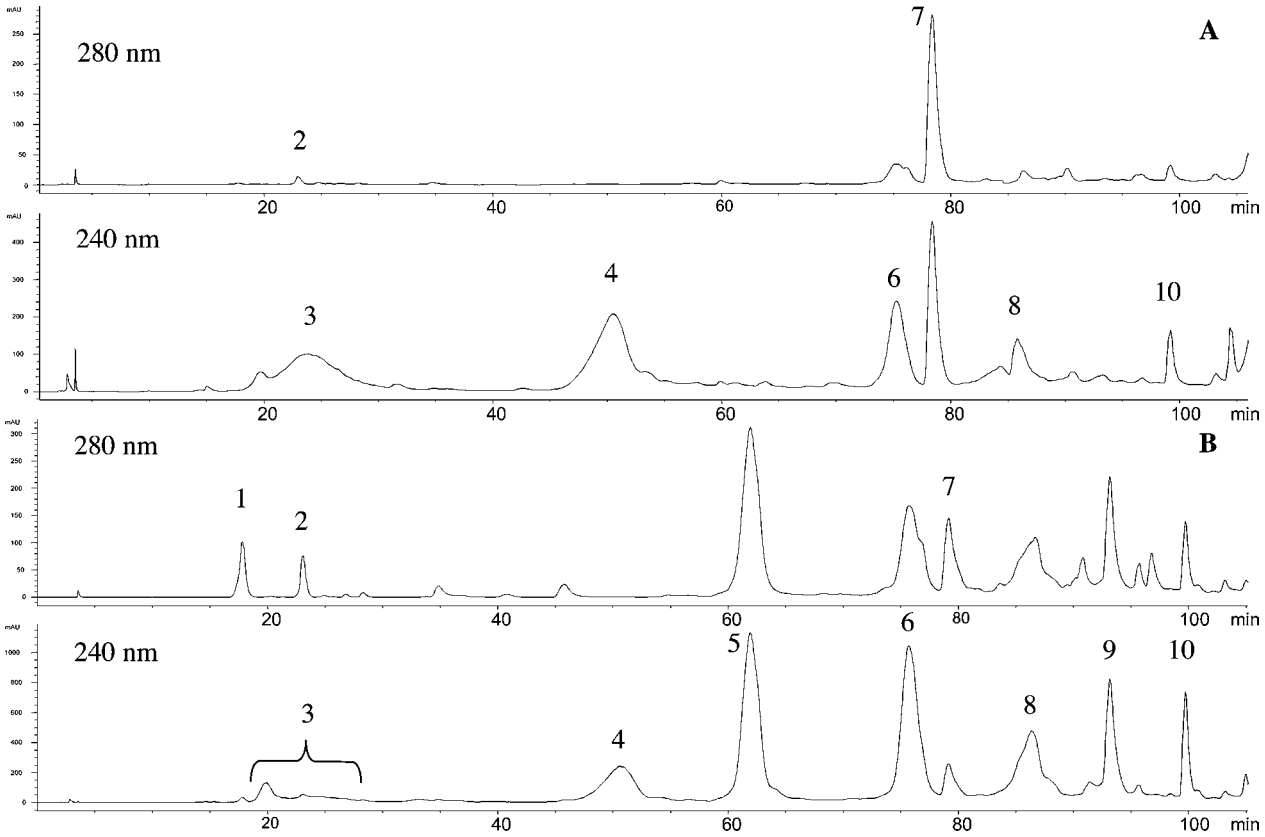

Figure 1. HPLC-DAD profiles of (**A**) Taggiasca virgin oil extract and (**B**) Seggianese virgin oil extract acquired at 280 and 240 nm. Identified compounds: 1, 5-hydroxytyrosol; 2, tyrosol; 3, elenolic acid derivatives; 4, elenolic acid; 5, deacetoxy-oleuropein aglycone; 6, oleocanthal; 7, acetoxypinoresinol; 8, oleuropein aglycone; 9–10, secoiridoids.

Table 1. Composition of Virgin Oil Extracts[a]

compound	Taggiasca oil, mg/L (%)	Seggianese oil, mg/L (%)
tyrosol	1.5 (0.7)	9.5 (2.1)
5-hydroxytyrosol	traces	14.0 (3.2)
5-hydroxytyrosol derivatives	ND	2.4 (0.5)
oleocanthal	8.3 (4.0)	53.0 (12.0)
elenolic acid	26.8 (12.9)	28.6 (6.5)
elenolic acid derivatives	15.8 (7.6)	6.6 (1.5)
deacetoxy-oleuropein aglycone	ND	102.1 (23.1)
oleuropein aglycones	ND	50.8 (11.5)
secoiridoid derivatives	3.6 (1.7)	51.0 (11.5)
lignan derivatives[b]	151.2 (72.5)	122.0 (27.6)
luteolin	1.3 (0.6)	1.9 (0.4)
total polyphenols	208.5 (100)	441.9 (100)

[a] Data reported are the mean of three determinations each performed in triplicate; SE was in the range 1–3%; ND, nondetectable. [b] Mainly acetoxypinoresinol.

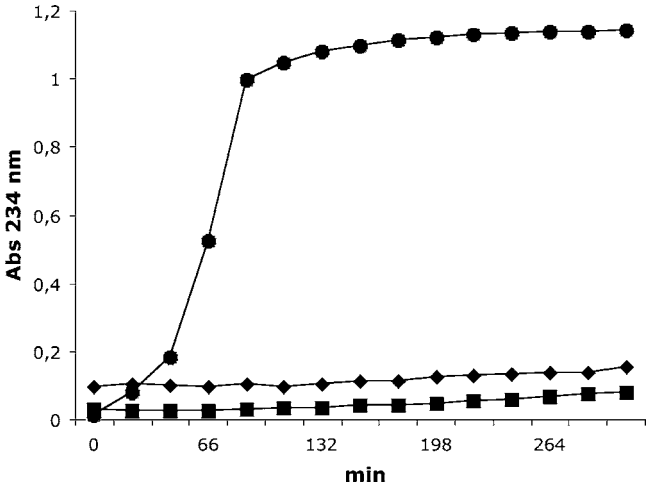

Figure 2. Typical profiles of conjugate diene formations induced by copper ions in human LDL (●); inhibitory effect of Taggiasca (◆) and Seggianese (■) oil extracts. Both Taggiasca and Seggianese extracts were used at a concentration of 5 μM as MPC.

factor in determining the MPC content. This observation is in line with earlier studies indicating that the cultivar, as well as agronomic and technological aspects of production, is related to MPC levels (*11*, *14*, *32*).

The two olive oil extracts examined in our study differ not only in total MPC levels but also in chemical composition. In the Taggiasca extract, the major compounds were lignans (72.5%) that are mainly constituted by pinoresinol derivatives (*45*), which seem to have a low antioxidant activity (*41*), whereas, in the Seggianese extract, the major compouds were secoridoids (deacetoxy-oleuropein aglycone, 23.1%; oleuropein aglycone, 11.5%; and secoridoid derivatives, 11.5%), which have high antioxidant activity. In addition, both oils contained different amounts of oleocanthal, an inhibitor of the prostaglandin-biosynthesis pathway (*34*) suggestive of anti-inflammatory activity, whereas flavonol levels did not differ substantially between the two oils. Interestingly, the two oil extracts also contain MPC that do not exert antioxidant activity (**Figure 3**), that is, tyrosol, elenolic acid, and derivatives of elenolic acid, and their total amounts are more elevated in Seggianese than in Taggiasca. Finally, the Taggiasca oil extract lacks 5-hydroxytyrosol, 5-hydroxytyrosol derivatives, deacetoxy-oleuropein aglycone, and oleuropein aglycone, whereas all of these compounds are present in the Seggianese oil extract.

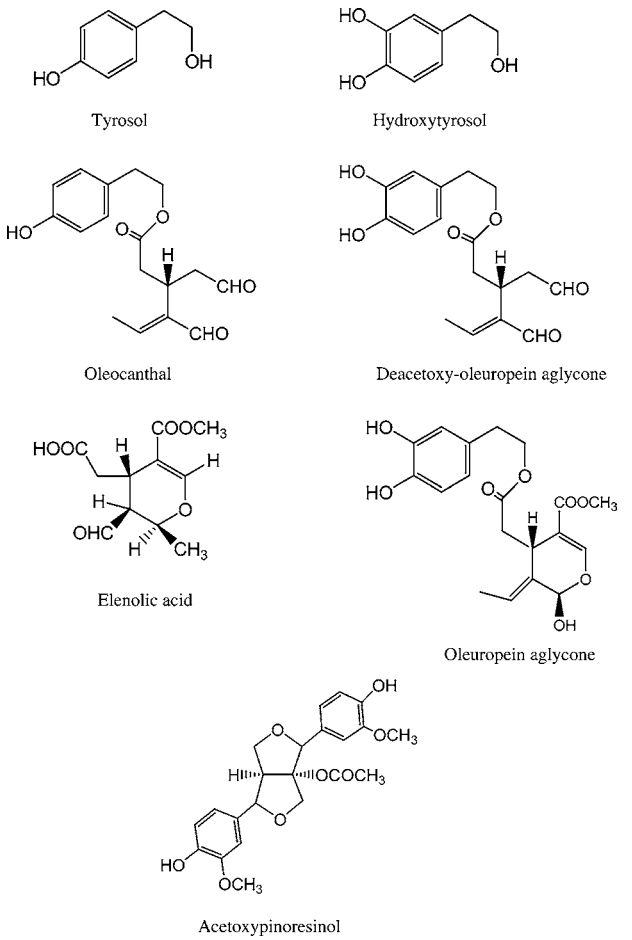

Figure 3. Structures of MPC.

Theoretically, the extract of Seggianese virgin oil, which has a greater amount of MPC and a lesser amount of MPC lacking in antioxidant activity, would exert a strong antioxidant effect. Indeed, the Seggianese oil extract was more potent (~4 times) compared with the Taggiasca oil extract. Importantly, the antioxidant effects and therefore the possible antiatherogenetic activities of the two virgin oils were dose-dependent and occur at low concentrations: IC_{50} values of 2.1 ± 0.9 and 0.6 ± 0.2 μM for Taggiasca and Seggianese oil extracts, respectively. After nutritional intake of virgin olive oil, the plasma level of MPC is ~0.6 μM (*46*), which is similar to the IC_{50} calculated for Seggianese oil extracts.

In conclusion, there are notable quantitative and qualitative differences between the two oil extracts studied, which could explain the differences observed in their antioxidant activities. In a small clinical study carried out with olive oils containing different amounts of MPC, the short-term inhibition of LDL oxidation is greater with oils rich in MPC (*30*). On the basis of this finding and our data, we suggest that clinical trials designed to evaluate the healthy properties of virgin olive oils should specify the type of virgin oil being tested and possibly its chemical composition, or at least the amount of MPC contained in the oil, so that results from different studies can be compared. It seems that the presence of 5-hydroxytyrosol and its derivatives and high levels of secoiridoids enhance antioxidant activities, suggesting that virgin olive oil rich in these compounds could have more potent health-protecting properties because the antioxidant activity of oils could have beneficial effect according to the oxidative hypothesis of atherosclerosis (*47*−*50*).

ABBREVIATIONS USED

MPC, minor polar phenolic compounds; LDL, human low-density lipoproteins; HPLC-DAD, high-performance liquid chromatography−diode array detection.

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