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Identification and Antioxidant Potential of Flavonoids and Low Molecular Weight Phenols in Olive Cultivar Chemlali Growing in Tunisia

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Increasing interest in phenolic compounds in olives is due to their antioxidant and health-enhancing properties. In this study the phenolics in fruits of the Tunisian olive cultivar Chemlali were extracted by methanol−water and fractionated using Sephadex LH-20 column chromatography. The identification of phenolic monomers and flavonoids was based on separation by high-performance liquid chromatography equipped with a diode array detector followed by liquid chromatography−mass spectrometry and gas chromatography−mass spectrometry analysis. Oleuropein, a secoiridoid glycoside esterified with a phenolic acid, was the major compound. Eight phenolic monomers and 12 flavonoids were also identified in Chemlali olives. Five flavonoids were isolated and purified using Sephadex LH-20 column chromatography and preparative paper chromatography. The antioxidant activity of the extract and the purified compounds was evaluated by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl and by using the β-carotene−linoleate model assay. Acid hydrolysis of the extract enhanced its antioxidant activity. Hydroxytyrosol and quercetin showed antioxidant activities similar to that of 2,6-di-*tert*-butyl-4-methylphenol. A hydroxyl group at the ortho position at 3′ on the B ring of the flavonoid nucleus could contribute to the antioxidant activity of the flavonoids.

KEYWORDS: Chemlali olive; phenolics; flavonoids; acid hydrolysis; antioxidant

INTRODUCTION

Olives (*Olea europaea* L.) and olive-derived products are an important part of the Mediterranean diet and recognized as a valuable source of natural phenolic antioxidants (*1*). The Chemlali olive is the most widespread and dominant olive cultivar in Tunisia (*2*). It occupies more than two-thirds of the olive plantations and contributes to >60% of the national olive oil production (*3*).

There is increasing interest in the phenolic compounds in olive products, and this is due to their biological properties (*4*). Most studies have focused on the composition of phenolics in olive oil using high-resolution techniques (*5*), and there is less information available on the phenolics of olive fruits (*6*, *7*). The compositions of olive fruit and olive oil exhibit some notable differences that are attributed to a series of chemicals and enzymatic alterations of some substances during oil extraction. These modifications include hydrolysis of glycerides by lipases, with formation of free fatty acids, hydrolysis of glycosides and oligosaccharides by glucosidases, oxidation of phenolic compounds by phenoloxidases, and polymerization of free phenols (*7*−*9*). Phenolics, such as simple phenols (monomers) and flavonoids, have been found in olive fruit (*10*). The most abundant compound in olive fruit is oleuropein. This constituent was routinely identified in the literature as the major phenol (*11*−*14*), although oleuropein is a secoiridoid glycoside, and therefore it is a terpenoid rather than a phenol. However, it is esterified with a phenolic acid, which gives it phenolic properties.

As a consequence of their fundamental chemical and antioxidant properties, the phenolics continue to attract considerable research effort. Lipid oxidation is known as one major factor in the deterioration of food during storage and processing. The addition of antioxidants has become popular in the food industry as a means of increasing the shelf life of products, as they prevent loss of sensory and nutritional quality. Several studies have shown that phenolic compounds reduce in vitro oxidation of low-density lipoprotein. Phenolics with multiple hydroxyl groups are generally the most efficient in preventing oxidation and, therefore, by inference, atherogenesis (*15*). Hence, much

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research has been conducted to find safe antioxidants with high activity from natural sources (*7*, *16*). Olive materials contain many compounds with antioxidant activity, mostly polyphenols, and could therefore be used as sources of potentially safe natural antioxidants for the food industry. The antioxidant capacities of oleuropein and hydroxytyrosol in *O. europaea* L. tissues are well-known (*4*), but few studies have been made on the antioxidant activities of other low molecular weight phenols, such as flavonoids, present in olive fruits.

The aim of this study is to identify, characterize, and evaluate the antioxidant potential of some flavonoids and simple phenolic compounds in the fruits of the Tunisian olive cultivar Chemlali.

MATERIALS AND METHODS

Plant Material and Extraction. Fruits from the olive variety Chemlali were obtained from a tree growing in Sfax, Tunisia. Samples were collected at the green maturation period (September 2002). Fruits (200 g) were selected randomly from around the tree and were then immediately blended into a homogeneous paste that was stored at 4 °C before extraction for 30 min. Five hundred milliliters of a mixture of methanol and water (4:1) was added to the olive paste, and this was left to stand overnight under agitation at room temperature. Subsequently, the solution was filtered using GF/F filter paper, and then the extract was washed two times with 300 mL of hexane in a separatory funnel. The extract was concentrated in vacuo at 40 °C and resuspended in water. The extract was redissolved in methanol and stored in glass vials, at 0 °C, and in darkness before chromatographic analysis.

Authentic Standards. *p*-Hydroxyphenylacetic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, tyrosol, and vanillin were obtained from Sigma-Aldrich (Chemie GmbH, Steinheim, Germany). Oleuropein was obtained from Extrasynthèse (Genay, France). Hydroxytyrosol was purified from olive mill wastewater as described previously (*17*). Apigenin, luteolin, luteolin 7-*O*-glucoside, quercetin, quercetin 3-arabino-glucoside, and rutin were obtained from Apin (Abingdon, U.K.). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and β-carotene were purchased from Fluka. Pure HPLC solvents were used in all cases.

Sephadex Column Chromatography. The methanolic extract of the olive fruits was fractionated on a Sephadex LH-20 column (2 × 60 cm, particle size = 25−100 μm; Pharmacia, Uppsala, Sweden) using methanol as the elution solvent. Fractions were collected using an LKB FRAC-100 collector (Pharmacia) and were analyzed by TLC (Kieselgel 60 F_{254}) with $CHCl_3/CH_3OH$ (80:20 v/v) as eluent. The eluted fractions were then pooled into eight major fractions, and they were further analyzed by HPLC-UV and LC-MS.

Purification by Means of Preparative Paper Chromatography. Crude fractions dissolved in 80% methanol were applied as streaks on sheets of Whatman no. 3 MM chromatography paper, which were run in *n*-butanol/acetic acid/water (4:1:5). After development and drying, the chromatograms were viewed under long-wave UV light (366 nm), and the fluorescent bands containing flavonoids were marked with pencil. These bands were cut out, cut into pieces (1 cm^2), and extracted in 80% aqueous methanol for 24 h. After filtration, the extracted fractions were concentrated and then chromatographed on Whatman no. 3 MM papers using 15 and 30% aqueous acetic acid as eluent. Flavonoid bands were marked, cut out, and eluted, as described above. The purity of the compounds was checked by HPLC.

HPLC Analysis for Phenolic Monomers. The identification of phenolic monomers was carried out by HPLC analysis. It was performed on a Shimadzu apparatus composed of an LC-10ATvp pump and an SPD-10Avp detector. The column was a C-18 (4.6 × 250 mm; Shimpack VP-ODS), and its temperature was maintained at 40 °C. The flow rate was 0.5 mL/min. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 50 min, and the following proportions of solvent B were used for the elution: 0−30 min, 20−50%; 30−35 min, 50%; and 35−50 min, 50−20%.

HPLC Analysis for Flavonoids. The HPLC system used to analyze the flavonoids consisted of a Waters LC 600 pump and a 996 photodiode array detector. The column employed was a Merck Lichospher 100 RP-18 (4 × 250 mm). A gradient of two solvents, A and B, was used. Solvent A consisted of 2% acetic acid in water and solvent B of methanol, acetic acid, and water (18:1:1). The following proportions of solvent B were used for elution: 0−20 min, 25−100%; 20−24 min, 100%; and 24−30 min, 25%. The column temperature was maintained at 30 °C, and the flow rate was 1 mL/min.

GC-MS Analysis. GC-MS was performed with an HP model 5872A, equipped with a capillary HP5MS column (30 m length; 0.32 mm i.d.; 0.32 μm film thickness). The carrier gas was He used with a 1.7 mL/min flow rate. The oven temperature program was as follows: 1 min at 100 °C, ramped from 100 to 260 °C at 4 °C/min, and 10 min at 260 °C. The injection mode was splitless. One hundred microliters of bis(trimethylsilyl)acetamide (BSTFA) was added to 100 μL of the ethyl acetate extract of hydrolysate products. The obtained solution was incubated for 20 min at 80 °C. Ethyl acetate and BSTFA were evaporated under a N_2 current in a fume hood, and the residue was redissolved in 1 mL of ethyl acetate and analyzed by GC-MS.

LC-MS Analysis. The LC-MS system used was a Waters apparatus composed of a 600 E pump, a Merck-Hitachi L-400 UV detector, and a Merck Lichrosphere 100 RP-18 column (4 × 250 mm). Positive-ion APCI-MS mode was obtained with a quadrupole ion trap instrument (Finnigan-MAT LCQ) as described previously (*18*). Identification of compounds by LC-MS analysis was carried out by comparing retention times and mass spectra of the unknown peaks to those of standards.

Colorimetric Determination of Total Phenols. The total phenols were determined according to the colorimetric reaction with Folin−Ciocalteu reagent (*19*). An aliquot of the olive fruit aqueous methanol extract (5 mL) was mixed with (2 mL) Folin−Ciocalteu reagent (Prolabo) and 35 mL of distilled water. After 3 min in the dark, 3 mL of sodium hydroxide solution (6% v/v) was added, and the mixture was shaken. The volume was adjusted to 50 mL by distilled water. The blue color formed was measured at 727 nm. The total phenols of the aqueous methanol olive extract and its hydrolysis, as determined by the Folin−Ciocalteu method, were reported as pyrogallol equivalents by reference to a standard curve ($y = 0.0017x$, $r^2 = 0.99$).

Acid Hydrolysis. Fifty milligrams of the olive extract was dissolved in 2 mL of a $MeOH/H_2O$ (4:1) mixture in a sealed vial. The solution was hydrolyzed at 100 °C for 1 h using 1 mL of a 2 M solution of HCl. After 1 h, the sample was cooled and diluted with water (1 mL), and the hydrophobic fraction was extracted by a separatory funnel three times with 3 mL of ethyl acetate, which was subsequently removed by evaporation.

Evaluation of Antioxidant Activity of Isolated Flavonoids by β-Carotene. In this experiment, scrupulous care was taken to avoid contamination by heavy metals. All experimental work was carried out using glass equipment to minimize metal contamination. All glassware was immersed for at least 24 h in EDTA (0.1%), rinsed several times with deionized water, and dried at 150 °C before use. The antioxidant activity of the isolated fractions was evaluated using a β-carotene−linoleate model system as described previously (*20*). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then pipetted into a round-bottom flask. After removal of chloroform under vacuum, using a rotary evaporator at 40 °C, 20 mg of linoleic acid, 200 mg of Tween 80 emulsifier (Aldrich Chemical, Co., Milwaukee, WI), and 50 mL of oxygenated distilled water were added to the flask, which was shaken vigorously. Aliquots (5 mL) of this prepared emulsion were transferred into a series of glass vials containing 200 ppm of each extract or 200 ppm of 2,6-di-*tert*-butyl-4-methylphenol (BHT) that was used as a positive control for comparative purposes. As soon as the emulsion was added to each vial, the zero time absorbance was read at 470 nm. Absorbance readings were then recorded at 15 min intervals until the control sample had changed color. The absorbance was then recorded at hourly intervals until the color of β-carotene in the experimental samples had disappeared. During the experiment all samples were kept in a water bath at 50 °C. Absorbance decreased rapidly in samples without an antioxidant, whereas in the presence of an antioxidant they retained their color, and thus absorbance, longer.

DPPH Radical Scavenging Assay. The DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging effect was evaluated according to

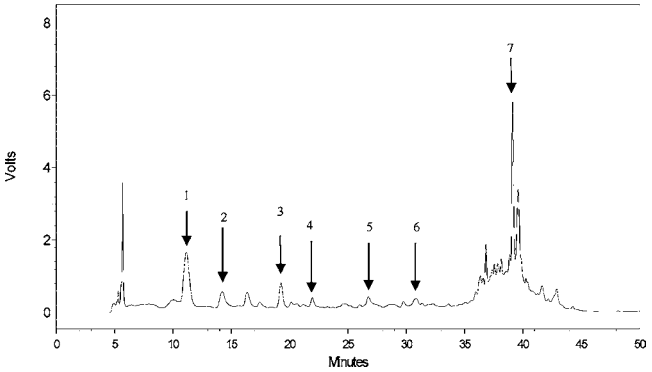

Figure 1. HPLC chromatogram of Chemlali olive extract at 280 nm. Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, *p*-hydroxyphenylacetic acid; 4, caffeic acid; 5, coumaric acid; 6, ferulic acid; 7, oleuropein.

Table 1. Abbreviated Mass Spectra of Major Phenolic Monomers Identified in Chemlali Olive Extract

TMS derivatives of	mass spectra (*m*/*z* and % of the base peak)
hydroxytyrosol	370 (M^+, 39); 267 (90); 193 (25); 179 (12); 73 (100)
tyrosol	282 (M^+, 18); 267 (13); 193 (15); 179 (100); 73 (42)
homovanillyl alcohol	312 (35); 298 (15); 267 (20); 209 (100); 193 (10); 179 (20); 73 (32)
3,4-dihydroxyphenyl-propionic acid	398 (M^+,100); 383 (12); 281 (15); 267 (75); 179 (98); 127 (15); 73 (75)
caffeic acid	396 (M^+, 100); 381 (25); 307 (12); 239 (11); 219 (92); 191 (13); 73 (55)
p-coumaric acid	308 (M^+, 81); 293 (100); 249 (44); 219 (82); 179 (13); 73 (51)
ferulic acid	338 (M^+,90); 323 (50); 308 (41); 293 (31); 267 (35); 249 (45); 147 (20); 73 (100)

the method employed by Na Mee et al. (*21*). Four milliliters of methanolic solution of varying sample concentration (25, 50, 100, and 150 μg/mL) was added to 10 mL of DPPH methanol solution (1.5×10^{-4} M). After the two solutions had been gently mixed and left for 30 min at room temperature, the optical density was measured at 520 nm using a Shimadzu UV-160 A spectrophotometer. The test samples and positive control BHT were tested over a range of concentrations The antioxidant activities of each test sample and BHT were expressed in terms of concentration required to inhibit 50% DPPH radical formation (IC_{50}, μg/mL) and calculated from the log-dose inhibition curve.

RESULTS AND DISCUSSION

Identification of Oleuropein and Phenolic Monomers. A reverse-phase HPLC technique was used to identify the major phenolic compounds in the extracts of olive fruits. The identification was based on comparisons of the chromatographic retention time and UV absorbance spectra of compounds in olive extracts with those of authentic standards. A representative chromatogram of the HPLC analysis of an olive extract is given in **Figure 1**. This chromatogram shows that the olive extract is made up of hydroxytyrosol, *p*-hydroxyphenylacetic acid, tyrosol, caffeic acid, *p*-coumaric acid, ferulic acid, and oleuropein, which was confirmed by GC-MS analysis (**Table 1**). In addition, two other phenolic compounds, homovanillyl alcohol and 3,4-dihydroxyphenylpropionic acid, were identified by GC-MS analysis. The obtained mass fragments agreed with those described previously (*22*). The identification of oleuropein was also confirmed by using an LC-MS apparatus in the positive mode. The spectrum exhibited a molecular ion at *m*/*z* 541 with fragments at *m*/*z* 137, 165, 225, 243, 361, and 379, which was consistent with the known fragmentation scheme for oleuropein (*23*).

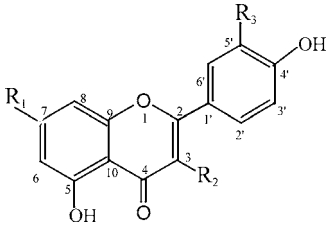

Figure 2. Structures of isolated flavonoids: **2**, luteolin 7-*O*-glucoside, R_1 = *O*-glucose, R_2 = H, R_3 = OH; **5**, rutin, R_1 = OH, R_2 = *O*-rutinose, R_3 = OH; **9**, quercetin, R_1 = OH, R_2 = OH, R_3 = OH; **10**, luteolin, R_1 = OH, R_2 = H, R_3 = OH; **11**, apigenin, R_1 = OH, R_2 = H, R_3 = H.

Purification and Identification of Flavonoids. To elucidate the structures of flavonoids in the green olive extract, the methanol extract was fractionated on a Sephadex LH-20 column, and the fractions were monitored by paper chromatography and HPLC with diode array detection. Paper chromatograms of the Sephadex fractions showed dark spots in UV having R_f values and colors specific for flavonoid aglycons and flavonoid glycosides as described previously (*24*), and also blue spots due to other phenolics. HPLC analysis of the fractions showed the presence of peaks with flavonoid-type UV spectra (two bands, λ_{max} of band 1 between 320 and 350 nm and λ_{max} of band 2 between 250 and 270 nm) and interfering peaks of other phenolics. The Sephadex fractions were purified using PPC to yield five pure flavonoids and a number of other flavonoids still in mixtures. The flavonoids were identified using a combination of HPLC with diode array detection and liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (APCI-LC-MS) (*15*) on the basis of their R_f values, UV spectra, and mass spectra and by comparison of the spectra with those of available authentic standards (**Table 2**). As the flavonoids were present at low concentrations it was not possible to isolate them in sufficient amounts for identification by NMR spectroscopy. The flavones [luteolin 7-*O*-glucoside (**2**), luteolin (**10**), and apigenin (**11**)] and flavonols [rutin (**5**) and quercetin (**9**)] were identified from their UV and mass spectra (**Figure 2**). **Table 2** lists each of the identified flavonoids in elution order. The structure assignment of flavonoids for which no standards were available was based on a systematic search for molecular ions using extracted ion mass chromatograms and comparing those with data in the literature (*11*, *25*, *26*). For example, the APCI mass spectrum in positive mode of compound **1** exhibited a base peak $[M + H]^+$ at *m*/*z* 595 and fragment ions at *m*/*z* 415, 385, and 355 (**Table 2**). No fragment was obtained of the flavonoid aglycon. This suggested that **1** might be a flavone *C*-glycoside. Losses of 180, 210, and 240 amu from the pseudomolecular ion to produce the ions at *m*/*z* 415, 385, and 355 are also characteristic of flavone di-*C*-glucosides, as during APCI-MS the glucose moieties are cleaved, each losing fragments of 90 and 120 amu (*18*), which added up to give losses of 180, 210, and 240 amu. The mass and UV spectral data of **1** and its retention time suggested that it was apigenin 6,8-di-*C*-glucoside (vicenin-2). This is the first report of vicenin-2 from cultivars of *O. europaea*, including the cultivar Chemlali. The pseudomolecular ion $[M + H]^+$ of compound **6** was also at *m*/*z* 595 (**Table 2**), but there was an intermediate ion at *m*/*z* 449 and an aglycon ion at *m*/*z* 287. The loss of 146 amu from the pseudomolecular ion represents the sugar rhamnose, and the loss of 162 amu from the intermediate ion is due to the loss of glucose. The λ_{max} of the UV spectrum at 351, 266 sh, and 255 nm suggests that flavonoid **3** is a luteolin 7-*O*-glycoside, and the results of the MS and UV spectra combined suggest that compound **3** could be luteolin 7-*O*-rhamnosyl-

Table 2. Flavonoids Detected in Chemlali Olives with Their HPLC Retention Times, UV Spectra, and Mass Spectral Data

no.	flavonoid	retention time(min)[a]	UV λ_{max} (nm)	$[M + H]^+$ (*m*/*z*)[b]	$[I + H]^+$ (*m*/*z*)[c]	$[A + H]^+$ *m*/*z*[d]
1	apigenin 6,8-di-*C*-glucoside (vicenin-2)	8.5	335, 271	595		
2	luteolin 7-*O*-glucoside	13.2	350, 267 sh, 255	449		287
3	luteolin 7-*O*-rutinoside	13.4	351, 266 sh, 255	595	449	287
4	quercetin 3-*O*-glucoside	13.6	355, 256	465		303
5	rutin	13.7	355, 256	611	465	303
6	apigenin 7-*O*-rutinoside	14.4	338, 266	579	433	271
7	chrysoeriol 7-*O*-glucoside	14.9	350, 267 sh, 253	463		301
8	luteolin 4′-*O*-glucoside	15.1	339, 268, 247 sh	449		287
9	quercetin	17.3	371, 255	303		
10	luteolin	18.0	351, 266 sh, 254	287		
11	apigenin	19.5	337, 267	271		
12	chrysoeriol	19.6	345, 267 sh, 252	301		

[a] For HPLC solvent system, see Materials and Methods. [b] APCI-MS (positive mode) data for the protonated molecular ion. [c] APCI-MS (positive mode) data for protonated intermediate molecular ions. [d] APCI-MS (positive mode) data for the protonated aglycon ion.

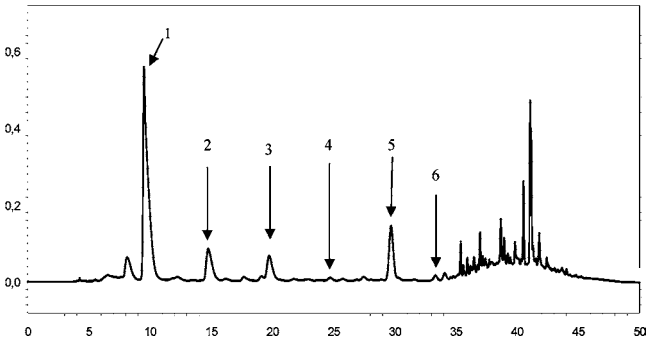

Figure 3. HPLC chromatogram at 280 nm of Chemlali olive extract after acid hydrolysis. Peaks: 1, hydroxytyrosol; 2, tyrosol; 3, *p*-hydroxyphenylacetic acid; 4, caffeic acid; 5, coumaric acid; 6, ferulic acid.

(1→2)glucoside (neohesperidoside) or the corresponding rhamnosyl(1→6)glucoside (rutinoside). The retention time of **3** is consistent with that of luteolin 7-*O*-rutinoside (that of the neohesperidoside is 2 min shorter). In the same way, flavonoid **6** was identified as apigenin 7-*O*-rutinoside and flavonoid **7** as chrysoeriol 7-*O*-glucoside. The mass spectrum of compound **8** was very similar to that of luteolin 7-*O*-glucoside, but the UV spectrum was that of a luteolin 4′-*O*-glycoside (*21*), and the much longer retention time was consistent with the identification of **8** as luteolin 4′-*O*-glucoside. The dominant flavonoids in green fruits of the Chemlali variety of olive sampled in September 2002 were flavonoid glycosides. Flavonoid glucosides and three aglycons (luteolin, apigenin, and quercetin) have been reported from Hardy's Mammoth olive cultivar (*11*). Owen et al. (*27*) detected only two flavonoid aglycons (apigenin and luteolin) in brined olive, and Vlahov (*25*) reported the identification of only flavonoid glycosides in fruits of olive cultivars.

Determination of Total Phenols. The analysis showed that the total amounts of phenols in the aqueous methanolic extract of the olive before and after acid hydrolysis were 9.63 and 12.54 g/kg of fresh olive fruits, respectively. The HPLC profile of phenols in the olive extract after hydrolysis showed a decrease of the amount of oleuropein and an increase of the levels of hydroxytyrosol and tyrosol (**Figure 3**). It has been reported that acid hydrolysis causes the degradation of oleuropein to produce hydroxytyrosol, glucose, and other oleuropein derivatives (*28*).

Antioxidant Potential of Crude Extract before and after Acid Hydrolysis. The DPPH radical scavenging activity of the olive extract before and after hydrolysis was measured and the IC_{50} determined as 2.5 μg/mL before and 1.9 μg/mL after hydrolysis. The increased antioxidant activity after hydrolysis

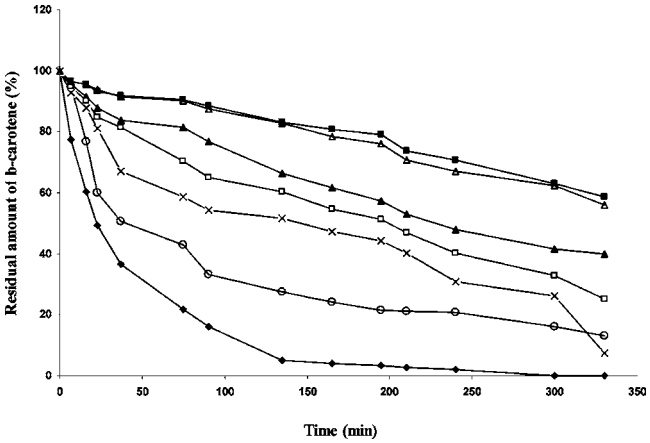

Figure 4. Oxidation of emulsified linoleic acid/β-carotene catalyzed by isolated flavonoids: (■) BHT; (△) quercetin; (▲) luteolin; (□) luteolin 7-*O*-glucoside; (×) rutin; (○) apigenin; (◆) control.

Table 3. Radical Scavenging Effect (IC_{50}) of Isolated Flavonoids

compound	IC_{50} (μg/mL)	compound	IC_{50} (μg/mL)
BHT	0.91	rutin	3.35
quercetin	0.93	luteolin 7-*O*-glucoside	3.40
luteolin	2.05	apigenin	33.84

may be due to the fact that the aglycons obtained after hydrolysis often have a higher antioxidant activity than their respective glycosides. Other mechanisms may also influence the antioxidant potential. For example, after hydrolysis, the increase in concentrations of hydroxytyrosol and tyrosol and the decrease in oleuropein could influence the antioxidant potential of the extract. The results suggest that the profile of phenolics in the extract rather than the total amount of phenolics influence the antioxidant activity of olive extracts.

Antioxidant Potential of Purified Flavonoids. The radical scavenging activities of five of the flavonoids [luteolin 7-*O*-glucoside (**2**), rutin (**5**), quercetin (**9**), luteolin (**10**), and apigenin (**11**)] isolated and purified from the olive extract are presented in **Figure 4** and **Table 3**. Two flavonoids, quercetin and luteolin, exerted strong antioxidant activities, which were comparable to that of BHT. Quercetin was the most effective. The flavonoids show similar trends of activity in both the linoleate model assay and the DPPH radical scavenging assay (**Figure 4**; **Table 3**). The flavonoids decreased in antioxidant activity in the order quercetin > luteolin > rutin = luteolin 7-*O*-glucoside > apigenin. A number of structural features seem to be important in determining the antioxidant trend. The foremost consideration

Table 4. Radical Scavenging Effect (IC_{50}) of Some Detected Simple Phenolics

compound	IC_{50} (μg/mL)	compound	IC_{50} (μg/mL)
BHT	0.91	*p*-coumaric acid	9.5
hydroxytyrosol	0.57	tyrosol	10.85
caffeic acid	0.88	*p*-hydroxyphenyl-acetic acid	12.20
oleuropein	1.19		

is the extent and nature of the hydroxylation pattern of the aromatic rings. For example, luteolin (3′,4′-dihydroxylation of the B ring) was a more powerful radical scavenger than apigenin (4′-monohydroxylation of the B ring) [IC_{50}(luteolin) = 2.05 μg/mL and IC_{50}(apigenin) = 33.84 μg/mL], suggesting that further hydroxylation at the C-3′ position contributes to a greater inhibition and increase of the antioxidant activity. Also, the hydroxylation of the C-3 of the non-phenolic C ring seems to be important, as the flavonol quercetin, which differs from the flavone luteolin in being 3-hydroxylated, has a stronger antioxidant activity than the latter compound (**Table 3**). Similar results have been reported previously on the inhibitory effects of several flavonoids on the thermal autoxidation of refined and deodorized palm oil (*29*, *30*). Furthermore, quercetin was a more potent radical scavenger than its glycoside, rutin, and luteolin had a higher activity than luteolin 7-*O*-glucoside.

All of the flavonoids assayed in the antioxidant test contain one or two hydroxyl groups (free or substituted) attached to the B ring of the flavonoid nucleus, and their potency appears to be affected by the level of substitution and the position of these groups on the ring. In this way, Rice Evans et al. (*31*) reviewed the biological properties of some flavonoids and focused on the relationship between their antioxidant activities, as hydrogen-donating free radical scavengers, and their chemical structures. Their results agreed well with our finding. Indeed, two flavonoids bearing free hydroxyl groups at the ortho position (quercetin and luteolin) showed the best antioxidative effects. Furthermore, an ortho hydroxyl group at position 3′ appears to be desirable to obtain an antioxidative response. For example, apigenin, which lacks this ortho hydroxyl group, shows very low antioxidant potential. In addition, the antioxidative action of most flavonoids may be partly the result of their chelating properties, and flavonoids such as quercetin and luteolin form strong binding complexes with metals, particularly with copper and iron (*29*, *32*).

DPPH Assay of Simple Phenolics. The antioxidative potential of simple phenolic compounds was also measured in the DPPH assay (**Table 4**). The presence of a single hydroxyl group (tyrosol, *p*-coumaric acid, and *p*-hydroxyphenylacetic acid having IC_{50} = 10.85, 9.5, and 12.20 μg/mL, repectively) confers a limited amount of antioxidant activity. On the other hand, the presence of an *o*-diphenol as in hydroxytyrosol and caffeic acid enhances the ability of the phenolic to act as an antioxidant. Hydroxytyrosol and caffeic acids showed IC_{50} values of 0.57 and 0.88 μg/mL, respectively, so they exhibited antioxidant activity at a level similar to that of BHT (IC_{50} = 0.91 μg/mL). In a previous study (*34*), we compared the effect of phenolic compounds in preventing the oxidation of β-carotene. We established a classification of these compounds according to their antioxidant potencies. In decreasing order, we have hydroxytyrosol = BHT > 3,4-dihydroxyphenylacetic acid > caffeic acid > *p*-hydroxyphenylacetic acid > tyrosol. It was clear that the antioxidant activity was related to the number and the nature of the hydroxylation pattern on the aromatic ring. It is generally assumed that the ability to act as a hydrogen donor and the inhibition of oxidation are enhanced by increasing the number of hydroxyl groups in the phenol. The solubility and partitioning behavior of the phenols will also influence their reactive activity.

Conclusion. This work is the first report on the identification and characterization of phenolic monomers and flavonoids in Chemlali olive fruits. The antioxidant activity of the olive extract was closely related to the chemical structure of the phenolic constituents. Acid hydrolysis caused an increase of the antioxidant potential. Five flavonoids were isolated and purified and studied for their antioxidant activities. Flavonoids such as quercetin and simple phenolic monomers such as hydroxytyrosol could be used as natural antioxidants and might substitute synthetic antioxidants that produce many undesirable secondary effects (*33*). In addition, phenolic compounds of olive are very important, as they contribute to sensory characteristics and the long stability of virgin olive oils, and they are also involved in biochemical and pharmacological effects. These compounds could make significant contributions to the health benefits associated with the consumption of olives.

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