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Comparative Study on Phenolic Content and Antioxidant Activity during Maturation of the Olive Cultivar Chemlali from Tunisia

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For the first time the identification and quantification of phenolic compounds of the *Olea europaea* L. cv. Chemlali olive were carried out to examine their profile during maturation. The phenolic composition was studied by using reverse-phase high-performance liquid chromatography during all steps of fruit development. Oleuropein is the abundant phenolic compound in Chemlali olive, and its concentration increases during maturation. An indirect relationship between oleuropein content in olive fruit and hydroxytyrosol was observed. Weak changes in the amounts of the other phenolic monomers and flavonoids were also observed. The total phenolic content varied from 6 to 16 g/kg expressed as pyrogallol equivalents. Its highest level was found at the last maturation period. The antioxidant capacity of olive extracts was evaluated by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl. The IC_{50} values of the olive extract ranged from 3.2 to 1.5 μg/mL. There was a correlation between antioxidant activity and total phenolic content of samples. The antioxidant activity increased with maturation. This could be attributed to the increase of the tolal phenol level with fruit development.

KEYWORDS: Olive; phenolic compounds; maturation; antioxidant

INTRODUCTION

Olea europaea L. is a typical tree widely cultivated for oil production in the Mediterranean area. Nowadays this region is the major international olive-growing area, accounting for almost 98% of the world's olive tree plantation (*1*). Tunisian olive plantations count about 57 millions trees covering 12623 ha. *O. europaea* L. cv. Chemlali is the most abundant olive variety, which represents >60% of the total olive trees in Tunisia (*2*). Olives are rarely consumed as a natural fruit due to their extreme bitterness. They are used for the extraction of oil instead and to a lesser degree as table olives.

It is well-known that the decreased incidence of cardiovascular disease in the Mediterranean area has been partly attributed to the consumption of olive products (*3*). These nutritional and medicinal qualities could be related to the phenolic compounds, which are considered to be responsible for conferring specific organoleptic and antioxidant proprieties to the olive derivatives (*4*). The interest in olive polyphenols is due to the fact that they may play a role in human diet and health. These compounds act mainly as antioxidants and radical scavengers and could be used as sources of potentially safe natural antioxidants for the food industry (*5*). Olive oil is known as the only stable oil during its storage and processing (*6*). The phenolic compounds increase the shelf life and nutritional quality of oil (*7*).

Several studies have focused on the phenolic content of olive oil using high-resolution techniques (*6*, *8*, *9*). However, little work was conducted on the phenolic composition of olive fruit (*1*, *10*). There are some notable differences in phenolic composition between the mature and immature fruit that are attributed to a series of chemical and enzymatic alterations of some phenolics during the maturation phase. These modifications include hydrolysis of glycosides by glycosidases, oxidation of phenolic compounds by phenol oxidases, and polymerization of free phenols (*11*). Olives attain their maximum fruit weight after 8 months following the flowering period. This is followed by physiological modifications and changes in fruit color with the appearance of the purplish black olive fruit indicating the end of olive morphology development (*12*). Changes in phenol content during fruit development are important, and it is desirable to have a maturation index that can be related to fruit composition.

Oleuropein is known to be the most prominent individual phenolic component of olive fruits and responsible for their intense bitterness (*10*, *12*−*14*). The concentration of oleuropein varied with olive variety and declined with physiological development of fruit (*12*−*14*). A relationship between oleuropein content in olive and other phenols such as certain flavonoids, demethyloleuropein, and hydroxytyrosol has been revealed in many varieties (*12*).

The aim of this study was the identification and quantification of phenolic compounds in Chemlali olive cultivar by using a reverse-phase high-performance liquid chromatography (HPLC) system. The evaluation of oleuropein, hydroxytyrosol, flavonoids, and other phenolics at different stages of maturation

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Table 1. Dates of Fruit Harvests and Dominant Fruit Color at the Respective Sampling Date Harvests

harvest	sampling date	days from first harvesting	fruit color
1	July 1, 2002	0	green
2	July 15, 2002	15	green
3	July 30, 2002	30	green
4	Aug 29, 2002	60	green
5	Sept 9, 2002	71	green
6	Sept 16, 2002	77	green with red spots
7	Sept 30, 2002	91	green with purple spots
8	Oct 20, 2002	111	purple
9	Nov 4, 2002	126	purple-black
10	Nov 17, 2002	139	purple-black
11	Dec 20, 2002	172	black
12	Jan 20, 2003	203	black
13	Feb 20, 2003	234	black

was elaborated. In addition, we studied the antioxidant potential of olive extract during all stages of maturation.

MATERIALS AND METHODS

Olive Preparation and Sampling Strategy. Cv. Chemlali olives were harvested from Sfax (Tunisia). The olive samples were collected from the beginning of fruit development (beginning of July) until the end of olive morphology (end of February) (**Table 1**). The olives (200 g) were selected randomly from different parts of an olive tree, and then they were immediately transferred to the laboratory and freeze-dried. After that, the olives were blended into a homogeneous powder, which was stored for extraction. The phenolic content of olive fruits was profiled as a function of fruit development, harvest date, and fruit color (green, spotted, purple, and black) (**Table 1**). This method is believed to be more suitable for such an investigation because different colored fruits are known to be chemically distinct, particularly with respect to phenolic compounds.

Extraction. Extreme care must be taken to ensure correct extraction, devoid of chemical and enzymatic modifications, which will invariably result in artifacts (*15*). Besides, the extraction method adopted for phenolic compounds must enable the extraction of phenolic compounds and must avoid chemical modification. The solvents most used to extract polyphenolic compounds from olives are methanol and mixtures of methanol and water (*16*). Olives (200 g) were blended and then extracted twice using methanol and once using a mixture of methanol/water 80:20 (v/v). Thus, a multiple extraction procedure is essential for quantitative analysis of phenolic compounds. The solution was stirred at 120 rpm for 24 h at room temperature. Then, it was filtered using GF/F filter paper and transferred to a 500 mL flask, which was evaporated to dryness. The residue was redissolved in methanol and stored in glass vials at 0 °C in darkness for chromatographic analysis.

Reagents and Standards. Phenolic compounds including benzoic acid, caffeic acid, coumaric acid, ferulic acid, tyrosol, vanillic acid, and vanillin were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Oleuropein was purchased from Extrasynthèse, Genay, France. Hydroxytyrosol was prepared as described by Allouche et al. (*17*). Luteolin, luteolin 7-*O*-glucoside, quercetin, rutin, and quercetin 3-arabino-glucoside were obtained from Apin. Apigenin was obtained from Sigma. All phenolic compound solutions were made in a mixture of methanol/water 80:20 (v/v). Double-distilled water was used in the HPLC mobile phase. Pure HPLC solvents were used in all cases.

High-Performance Liquid Chromatography. Phenolic monomer and flavonoid identifications were carried out by HPLC analysis. It was performed on a Shimadzu apparatus composed of an LC-10ATvp pump and an SPD-10Avp detector. The columns used to analyze monomeric phenols and flavonoids were, respectively, a C-18 (4.6 × 250 mm) Shim-pack VP-ODS and a C-8 (4.6 × 250 mm) Shim-pack CLC. The temperature was maintained at 40 °C. 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. The elution conditions applied for monomeric phenols were as follows: 0−25 min, 10−25% B; 25−35 min, 25−80% B; 35−37 min, 80−100% B; 37−40 min, 100% B. Finally, washing and reconditioning steps of the column were included (40−50 min) with a linear gradient of 100−10% B. The flow rate was 0.6 mL/min, and the injection volume was 50 μL. For the elution program applied for flavonoids, the following proportions of solvent B were used: 0−20 min, 25−70%; 20−25 min, 70%; and 25−50 min, 70−25%. The flow rate was 1 mL/min, and the injection volume was 40 μL. All gradients used in this study provided adequate separation. The C-18 column, being more suitable for the resolution of the range of phenols, was used for routine phenolic profiling at 280 nm (*18*). The C-8 column provided adequate separation of flavonoid profiling, detected via UV monitoring at 335 nm (*19*). The identification and quantification of phenolic compounds in *O. europaea* L. cv. Chemlali olive were based on the HPLC spectra, on the retention time in comparison with phenolic standards analyzed in the same condition, and on the method of standard addition to the samples.

Total Phenol Measurement. The total phenols were determined by the colorimetric reaction with Folin−Ciocalteu reagent (*20*). An aliquot of the olive fruit aqueous methanol extract was mixed with 2 mL of Folin−Ciocalteau reagent (Prolabo). A sodium hydroxide solution (6% v/v) was added, and the mixture was shaken. The blue color formed was measured at 727 nm. The total phenols of aqueous methanol olive extracts, as determined by the Folin−Ciocalteu method, were reported as pyrogallol equivalents by reference to a standard curve ($y = 0.0017x$, $r^2 = 0.99$).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Effect. The DPPH radical scavenging effect was evaluated according to the method employed by Na mee et al. (*21*). Four milliliters of methanolic solution of different sample concentrations was added to a 10 mL DPPH methanol solution (1.5×10^{-4} M). After the two solutions had been gently mixed and left at room temperature for 30 min, the optical density was measured at 520 nm using a Shimadzu UV-160 A spectrophotometer. The antioxidant activity of each product and sample was expressed in terms of IC_{50} micrograms per milliliter concentration required to inhibit 50% of DPPH radical formation and calculated from the log-dose inhibition curve.

RESULTS AND DISCUSSION

Oleuropein and Monomeric Phenols. Fifteen phenolic compounds were identified in *O. europea* L. cv. Chemlali. These included classes of phenolic compounds such as hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and phenolic alcohols. These phenolic compounds and their respective retention times were as follows: hydroxytyrosol (11.2 min), tyrosol (17.6 min), *p*-hydroxybenzoic acid (19.9 min), vanillic acid (23.6 min), caffeic acid (25.8 min), coumaric acid (29.5 min), vanillin (34.2 min), ferulic acid (36.8 min), oleuropein (39.4 min), rutin (8.4 min), quercetin 3-arabino-glucoside (9.7 min), luteolin 7-*O*-glucoside (10.9 min), quercetin (18.3 min), luteolin (18.8 min), and apigenin (22.3 min). An external standard method was used to measure the concentrations as described by Chamkha et al. (*18*). All calibration curves were linear over the concentration range tested with correlation coefficients >0.96.

Figure 1 shows that a high concentration of oleuropein was obtained during the green maturation sampling. It attained 6.5 g/kg (all data are reported on a fresh mass basis) for the sample harvested on August 30. This concentration was far in excess in comparison with the other phenolics. However, the dominance of oleuropein was not sustained during the sampling period at various times. The content at initial harvest was 2.5 g/kg and then increased between the second and fourth harvests (**Table 1**). After this period, oleuropein concentrations decreased as the sampling period progressed and reached a minimum at the last stage of maturation (1.5 g/kg) (**Figure 1**). The change in oleuropein content may be related to the increased activity of hydrolytic enzymes during maturation (*13*, *14*). An indirect

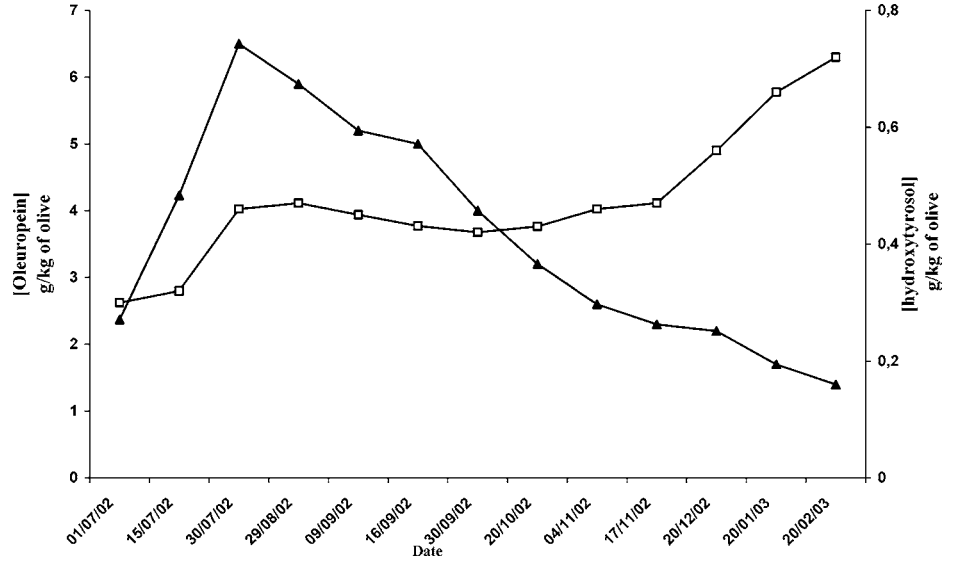

Figure 1. Oleuropein (▲) and hydroxytyrosol (□) levels during the maturation of olive variety. Each point represents the mean of two determinations and two independent experiments (SD < 15% of the mean).

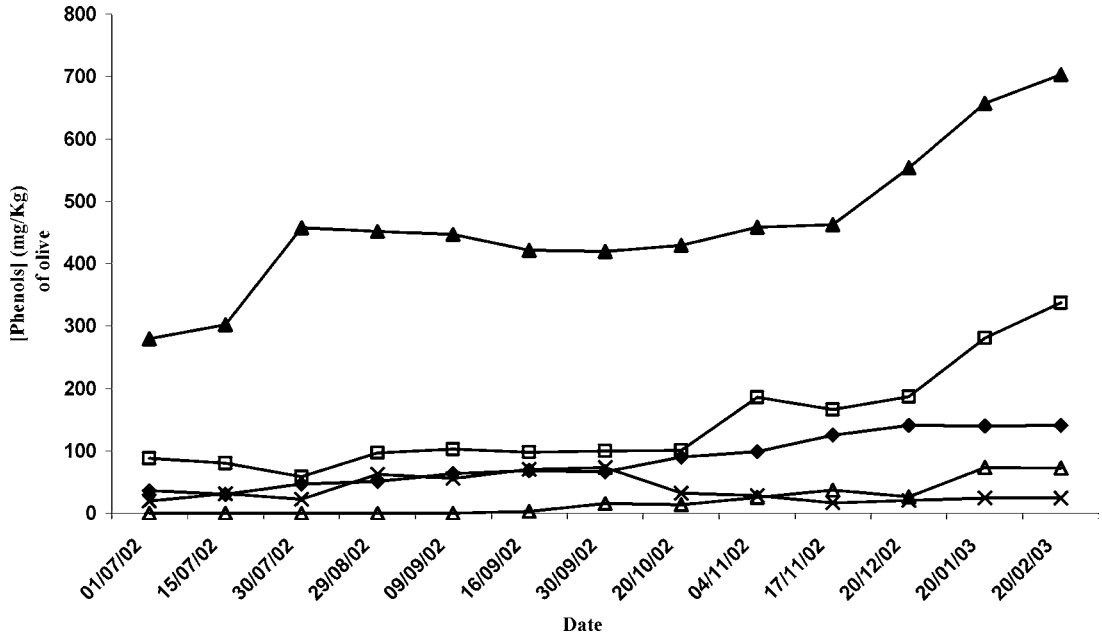

Figure 2. Concentration–time profile during maturation: (▲) hydroxytyrosol; (□) tyrosol; (◆) ferulic acid; (×) *p*-coumaric acid; (△) caffeic acid. Each point represents the mean of two determinations and two independent experiments (SD < 12% of the mean).

relationship between oleuropein content in olive fruit and hydroxytyrosol was observed from the end of October to the end of February. Indeed, the hydroxytyrosol concentration began at relatively low levels and increased as the sampling period progressed (**Figure 1**). Chemically, oleuropein is the ester of oleoside 11-methyl ester and 3,4-dihydroxyphenylethanol (hydroxytyrosol). Hydroxytyrosol is the principal product of oleuropein degradation during the maturation of fruit. Many chemical and enzymatic reactions cause the decrease of oleuropein concentration and the increase of hydroxytyrosol concentration (*11*). It was reported that glycosidase activities produced hydroxytyrosol, glucose, and other derivatives from oleuropein (*22*−*24*). Our results agreed with the findings of Amiot et al. (*12*, *13*), who observed a decline in oleuropein content with fruit maturity. Its levels vary from 3.3 to 45 g/kg and from 2.8 to 40 g/kg of olive fresh mass in cultivars Cailletier and Salonenque, respectively. Similarly, it was reported that oleuropein contents in the fruit of both cultivars Gentile (Larino) and Gentile (Colletorto) varied from 0.87 to 1.45 g/kg and from 1.5 to 2.08 g/kg, respectively (*14*). Bianco et al. (*25*) claimed that the concentration of oleuropein falls to zero when olive fruits (*O. europaea* cv. Leccino) are completely black.

HPLC analyses showed that other phenolic compounds were present during the maturation stages of olive, and their amounts are higher in black olives than in green ones (data not shown). **Figure 2** shows that tyrosol is the second most abundant phenolic monomer compound after hydroxytyrosol. The tyrosol concentration increased from 75 to 375 mg/kg during fruit maturation. This increase may arise from ligistroside transformation or the hydrolysis of other compounds containing tyrosol.

Many hydroxycinnamic acids were identified in this study. They included ferulic, caffeic, and *p*-coumaric acids. Ferulic acid remained relatively constant, although it increased slightly when the olives became black. *p*-Coumaric acid showed a weak variation and decreased in the last phase of maturation. Caffeic acid was present at a very low concentration during all phases

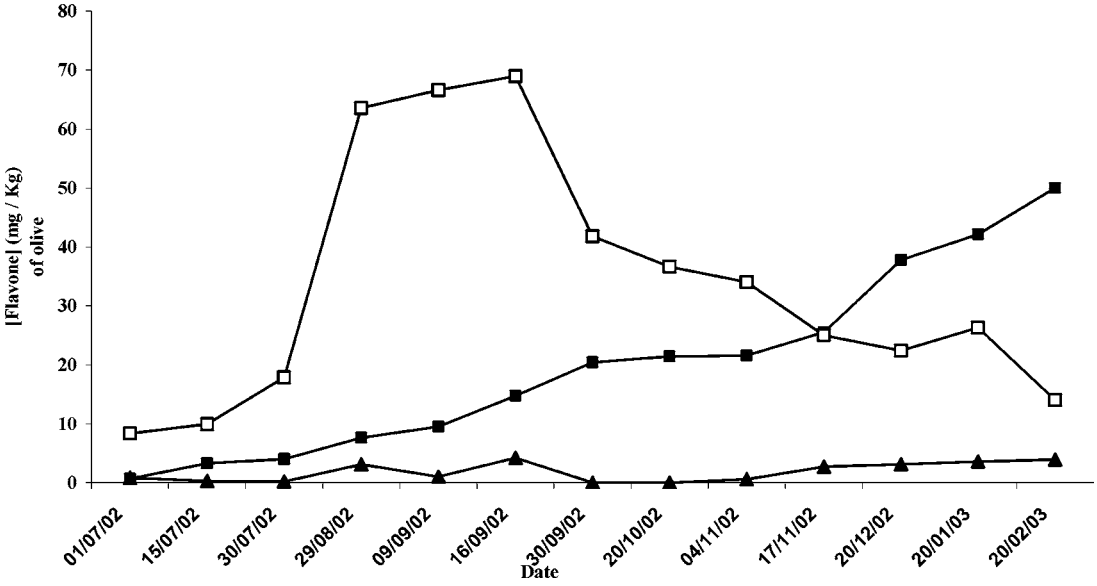

Figure 3. Evolution of the main flavone compounds (milligrams per kilogram of fresh fruit) during development and maturation of olive fruit: (□) luteolin 7-*O*-glucoside; (■) luteolin; (▲) apigenin. Each point represents the mean of two determinations and two independent experiments (SD < 15% of the mean).

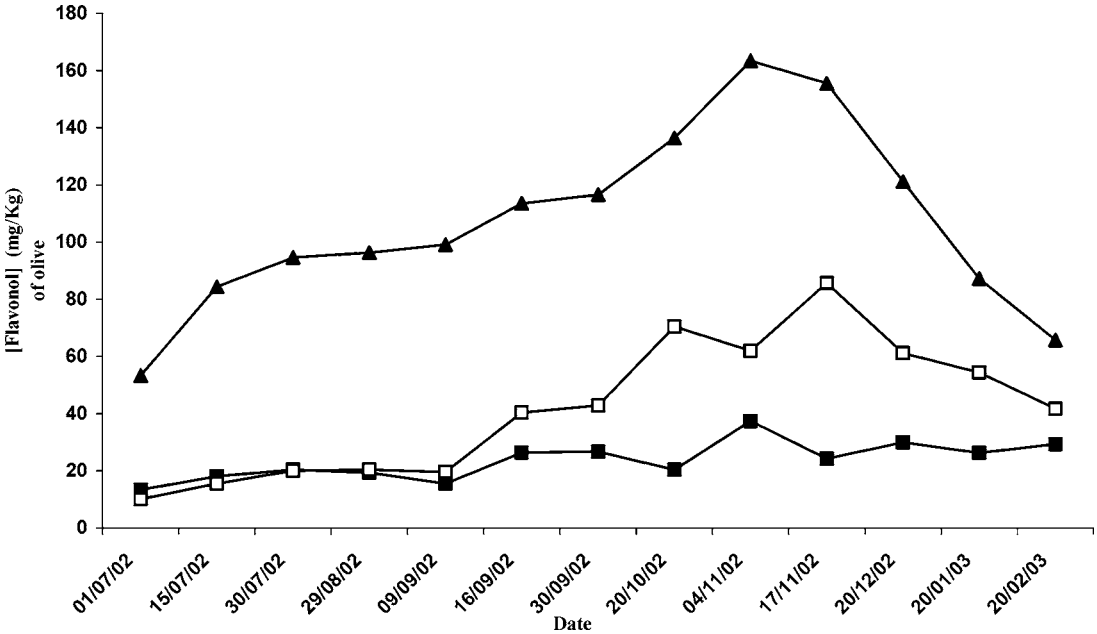

Figure 4. Evolution of the main flavonol compounds (milligrams per kilogram of fresh fruit) during development and maturation of olive fruit: (□) rutin; (■) quercetin; (▲) quercetin 3 arabino-glucoside. Each point represents the mean of two determinations and two independent experiments (SD < 15% of the mean).

of maturation (**Figure 2**). Other phenolic monomers including *p*-hydroxybenzoic acid, vanillic acid, and vanillin exhibited low concentrations and were present as minor components in the harvest samples. This is in agreement with findings about Spanish olives reporting that vanillic acid, vanillin, and *p*-coumaric and ferulic acids practically remained constant and that their concentrations were lower than 2−4 ppm during maturation (*6*).

Flavonoids. Six flavonoids were identified and quantified, and their quantities were reported in milligrams per kilogram of fresh weight of olives (**Figures 3** and **4**). The flavonoids studied were divided in two groups: flavones (luteolin 7-*O*-glucoside; luteolin and apigenin) and flavonols (rutin; quercetin 3-arabino-glucoside and quercetin). It appears that flavonoid glucosides were dominant and exhibited the most significant variation with physiological maturity. The main flavonoid compounds quantified in our study were luteolin 7-glucoside, rutin, and quercetin 3-arabino-glucoside. The luteolin 7-*O*-glucoside amount was noticeably higher between the fourth and sixth harvests, and the maximum concentration was reached in mid-September at a value of 70 mg/kg of fresh fruit (**Figure 3**). After this period, its concentration decreased until the end of the sampling period. At the same time, luteolin concentrations started at very low levels and increased as the sampling period progressed (**Figure 3**). The chemical structures of luteolin 7-glucoside and luteolin and their respective levels during fruit maturation suggest that they may be related biochemically. Thus, one possibility is that luteolin is a product of luteolin 7-*O*-glucoside transformation by glycosidase activities. Apigenin remains substantially unchanged and quite low throughout all

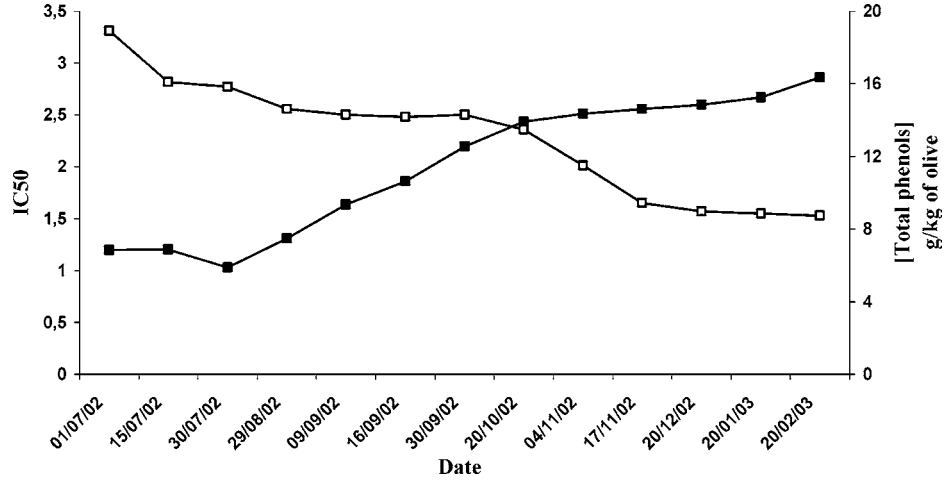

Figure 5. Correlation between total phenol concentration and DPPH radical scavenging effect (IC_{50}) of the extracts during maturation of fruits: (■) total phenol concentrations; (□) IC_{50}. Each point represents the mean of two determinations and two independent experiments (SD < 10% of the mean).

stages of maturation (**Figure 3**). These results correlate with previous findings reporting that luteolin may originate from rutin or luteolin glucoside and apigenin from apigenin glucosides and show opposite evolutions with their glucoside form derivatives (*6*).

Profiles of the three flavonols identified in olive during maturation were dominated by quercetin 3-arabino-glycoside. The last flavonol reached its maximum level at the 9th harvest and then decreased during the last stage of maturation (**Figure 4**). Rutin concentration increased slowly until the 10th harvest (80 mg/kg) and decreased in the end of the growing period of the fruit. Quercetin concentrations were noticeably lower and showed very little change during the maturation (**Figure 4**). This may be explained by the fact that *O. europaea* L. fruit appears to accumulate only glycosylated derivatives because they are probably less toxic than aglycons (*13*).

Total Phenol Determination. Figure 5 shows that the total phenol concentration of olive extracts varied from 6 to 16 g/kg of equivalent pyrogallol during maturation of the cv. Chemlali. The highest amount of the total phenols was present in black olives (from the harvest at the end of October to the end of February). This seems to corroborate the high concentration of phenolic compounds quantified by HPLC (simple phenols and flavonoid aglycons) during this same period of harvest. The lower amount determined by HPLC may be due to (i) a lack of quantification of all phenolic compounds detected in the chromatograms, (ii) the fact that many phenolics are not detected by HPLC in our experimental condition, and (iii) the presence of interactions between Folin reagent and hydroxyl groups of other compounds, such as proteins and sugars as was reported previously (*18*).

Antioxidant Potential of Crude Extracts during Maturation. The DPPH radical scavenging effect for all of the methanol extracts is shown in **Figure 5**. All extracts exhibited antioxidant activity, which showed correlation between total phenol content and DPPH radical-scavenging activity over all stages of maturation. The lower IC_{50} values (1.4 μg/mL), which indicated higher antioxidant potential, were observed for the samples harvested at the end of February. This is probably due to the significant radical inhibition caused by a high concentration of *o*-diphenol such as hydroxytyrosol and luteolin. The antioxidant activity and the level of the total phenols in the extracts suggest that the radical scavenging effect in the extract can be attributed to hydroxylated phenolic compounds, in particular, the number of hydroxyl substituents in the aromatic ring and the nature of the substituents at the para or ortho position (*26*). These compounds react with free radicals formed during autoxidation and generate a new radical that is stabilized by the resonance effect of the aromatic nucleus (*27*).

In conclusion many phenolic compounds were identified and quantified in Chemlali olives from Tunisia by using a reverse phase HPLC system. Oleuropein, the abundant phenolic compound, hydroxytyrosol, many flavonoids, and simple phenols vary quantitatively during maturation. The antioxidant potential of olive extract was found to increase during maturation in correlation with the total phenol content. The harvest at the last phase of maturation led to a decrease of the bitter taste of olive and an increase of the level of flavonoid aglycons and hydroxytyrosol. These phenolic compounds of olives are of great interest, as they contribute to sensory characteristics and the long stability of virgin olive oils and as they may be involved in biochemical and pharmacological effects, including anticarcinogenic and antioxidant properties (*3*). The phenolic composition represents a useful contribution to the biochemical characterization of the Chemlali olive cultivar. Further studies of the effects of many environmental factors and growing conditions on the phenolic composition of Chemlali olives are under investigation.

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