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The potential of wild olive leaves (*Olea europaea* L. subsp. oleaster) addition as a functional additive in olive oil production: the effects on bioactive and nutraceutical compounds using LC–ESI–QTOF/MS

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Abstract

This study aims to investigate the influence of traditional maceration upon the enrichment of olive oil with oleaster leaves. The phenolic and tocopherolic compositions of control olive oil and enriched olive oils were determined. The influence of these oil preparation procedures on oil quality indicators was also investigated through spectrophotometric indices and fatty acid profiles. The total contents of bioactive compounds and pigments improved in oils obtained by maceration of fresh wild olive leaves, and were in statistically significant correlation with leaves proportions additions. The obtained results revealed that 15 phenolic compounds belonging to different phenolic types were characterized and quantified by an effective HPLC–DAD–ESI–MS/MS method. In all expected olive oils, the oleuropein aglycon (3,4-DHPEA-EA), and ligstroside aglycon (*p*-HPEAEA) derivatives were the most abundant compounds. Similarly, to phenolic compounds, tocopherols strongly increased with leaves addition during maceration process.

The data obtained from this study suggested that the addition of olive leaf to oils allowed more functional olive oils with higher antioxidant contents. Thus, Extra Virgin Olive Oil (EVOO) extracted with 10% of olive leaves presented the highest amount of phenolic and tocopherol compounds.

Keywords Olive oil · Wild olive leaves · Maceration · HPLC–DAD–ESI–MS/MS · Phenolic compounds · Tocopherol

Introduction

Olive oil is the main lipid source in the diet of the Mediterranean countries and is related to diverse healthy benefits based upon its richness in monounsaturated fatty acids and its high concentration of minor phenolic compounds has been linked to a variety of health advantages [37]. Olive oil's oxidative stability is connected to its endogenous phenolics, which are a key class of secondary metabolites with high antioxidant activity [38].

In the food industry, the concept "superfoods" is being used to describe foods which purport to have health advantages [24]. In the last 2 decades, the food industry has been seeking for additives to boost the therapeutic benefits of food items [25]. Several additives and active chemicals from various sources are being researched as antibacterial, anti-inflammatory, and possible antiviral agents to keep up with this tendency [24].

Furthermore, the food sector is contemplating the new phase following the COVID-19 pandemic, wherein consumers are interested in consuming items that will help them strengthen their immunity system and eat healthier [25].

Accordingly, the extraction of bioactive compounds for the development of functional meals becomes a barrier, necessitating the identification of potent natural component sources to enhance the availability of healthier food choices. In this regard, one method to valorizing the enormous spectrum of bioresources is for the food industry to investigate innovations that disrupt the way we eat food [25], [33].

Various studies have recently proved the utility of natural antioxidants as oxidation inhibitors in edible oils [33, 41].

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Previous research has looked at enriching edible oils, such as olive oil, using olive leaf extracts to increase its organoleptic quality and oxidative stability. Also, Marx et al. [33] signaled that olive leaves are rich in phenolic compounds and their extract has an antioxidant potential. Olive leaves are commonly considered as a byproduct with a lot of opportunity for value addition [1, 33]. Phytochemicals from olive leaves can be recovered for valorization in food items, resulting in high added-value compounds [33].

In the same regard, olive leaves are a rich source of phenolic compounds (such as oleuropein, verbascoside, apigenin-7-glucoside, luteolin-7-glucoside, and others) [14, 19], which may defend against free radicals assaults through several mechanisms (antioxidant, signaling, etc.) [33]. Furthermore, olive leaves are one of the most prevalent by-products of the olive oil industry (counting to 10% of total weight of olives) (Vidal et al. 2019) and may be exploited as a low-cost source of high-added-value phenolic compounds. Caponio et al. [19] also pointed out that the pharmaceutical, cosmetic, and food sectors are becoming more interested in the therapeutic effects attributed to these bioactive components in olive leaf extract. The antibacterial and antioxidant properties of the phenolic compounds found in olive leaves suggest that they might be used as natural additives or supplements [2], [33].

On the other front, the extraction procedure is crucial for obtaining a high phenolic recovery from the samples. The extraction of the bioactive compounds requires efficient techniques, but only a few studies show good extraction yields using clean techniques [17, 33]. Maceration extraction is a healthy method for the extraction of bioactive compounds [33]. Maceration is a traditional extraction method that has been used to extract phenolic chemicals from plants for a long time [26, 33].

Olive leaf extract has become more popular in recent years for usage in meals, food additives, and functional food components [2, 33]. Accelerating efforts to establish sustainable and contemporary food systems, involving large food supply chains based on by-products and their re-use in the food industry, is worthwhile [36]. The chemical composition of olive leaves is known in details, nevertheless, most of the research have been concerned with material derived from cultivated olive trees, while studies dealing with wild types of *Olea europaea* are limited. Furthermore, the antioxidant properties of leaves extract from wild olive trees with greater bioactive content [6] have not been well explored. Thus, the aim of our study was to qualitatively and quantitatively evaluate wild olive leaves addition on olive oil nutraceutical compounds by focusing on the major groups of phytochemicals such as phenolic, fatty acids, and tocopherol oil composition [36].

The present research was undertaken to evaluate and compare the phenolic and tocol profiles of control and enriched olive oil by wild olive tree leaves using various percentages maceration.

Materials and methods

Plant material

Olive fruits from Chemlali variety were hand-picked at maturity index 3.5 during the crop season 2020–2021. Oil extraction was carried out in similar industrial conditions using a laboratory instrument: Abencor analyzer (MC2 Ingenieriay Sistemas, Sevilla, Spain) consisting of three basic elements: a mill, a thermobeater and a pulp centrifuge. After harvesting, fresh olive samples were washed, deleafed and then crushed with a hammer mill and were slowly mixed for 30 min at 25 °C. The obtained paste was centrifuged without addition of warm water. The oil was separated by decanting. Then, wild olive leaves (0%, 1%, 3%, 5%, and 10% by weight) were placed in 250 ml glass bottles with hermetic closure and to these the oil was added until immersed and left for 7 days to infuse. A control sample was obtained using only olive oil. The fortified oils were stored in the dark at 4 °C until the extraction and analysis.

Chemicals

Hydroxytyrosol, tyrosol, vanillin, vanillic acid, ferulic acid, *p*-coumaric acid, cinnamic acid, luteolin, and apigenin were used as reference standards for the optimization of the extraction procedure and purchased from Sigma–Aldrich (Schnelldorf, Germany). Methanol (HPLC-grade quality) was used for the extraction (Baker, Avantor Performance Materials, Arnhem, Netherlands). For HPLC–Qtof–MS analysis formic acid (Honeywell Fluka, Fisher Scientific, Schwerte, Germany), methanol (Fisher Scientific, Schwerte, Germany), isopropanol (99.9%, Honeywell, Riedel-de-Häen, Fisher Scientific, Schwerte, Germany), 1 M sodium hydroxide solution (Agilent Technologies, Santa Clara, CA, USA), and water (Merck, Darmstadt, Germany) were used (HPLC-grade quality).

Petroleum ether (40–60 °C analytical grade > 98%), heptane, tocopherols standards, sodium methylate, sodium hydrogen sulphate (monohydrate, extra pure), and tert-butyl methyl ether (HPLC grade) were purchased from Merck (Darmstadt, Germany). Tocopherol and tocotrienol standard compounds were purchased from CalBiochem (Darmstadt, Germany)

Quality index determination

Free acidity and specific UV spectrophotometric indices at 232 and 270 nm were carried out following the analytical methods described in the European Union Commission Regulations EEC/2568/91 and EEC/1429/92 [22].

UV absorbency is an indicator of oxidation, especially in oils. It measures the quantity of certain oxidized compounds that absorb wavelengths of 232 and 270 nm.

Free acidity, given as percent of oleic acid, was determined by titration of a solution of oil dissolved in ethanol/ether (1:1, v/v) with 0.1 mol/l (0.1 N) potassium hydroxide solution. Peroxide value expressed as milliequivalents of active oxygen per kilogram of oil (meqO$_2$/kg), was determined as follows: olive oil (2.5 g) was dissolved in a mixture of chloroform/acetic acid (2:3, v/v) and was left to react with a solution of potassium iodide in the darkness; the free iodine was then titrated with a 0.01 mol/l sodium thiosulfate solution. K_{232} and K_{270} extinction coefficients were calculated from absorption at 232 and 270 nm, respectively, with a UV spectrophotometer (Model SECOMAM ANTHELIE Advanced, France), using a 1% solution of oil in cyclohexane and a path length of 1 cm (IOOC,2019).

Chlorophyll and carotenoid contents

According to the method of Minguez-Mosquera et al. [34], carotenoid and chlorophyll compounds were determined at 470 and 670 nm, respectively, in cyclohexane, using the specific extinction coefficients. The values of the specific extinction coefficients used were E0 = 613 for pheophytin as major component in the chlorophyll fraction and E0 = 2000 for lutein as major component in the carotenoid fraction. Thus, pigment contents were calculated as follows:

$$\text{Chlorophylls}\left(\text{mg kg}^{-1}\right) = \frac{A_{670 \times 10^6}}{613 \times 100 \times d},$$

$$\text{Carotenoids}\left(\text{mg kg}^{-1}\right) = \frac{A_{470 \times 10^6}}{2000 \times 100 \times d},$$

where A is the absorbance and d is the spectrophotometer cell thickness (1 cm).

Carotenoids and chlorophylls contents were expressed as mg of lutein and pheophytin ''a'' per kg of oil, respectively.

Radical-scavenging activity (RSA)

The DPPH antioxidant test was used to determine the radical-scavenging activity of the samples. The free radical-scavenging activity (RSA) was determined using a modified Goupy et al. technique (2012).

Briefly, the oil extracts were prepared by diluting 0.5 g of oil in 10 mL of methanol. All solutions were prepared freshly for each analysis. 1 ml of each solution was mixed with 2 ml of DPPH solution (2×10^{-4} mol/L in methanol) and kept at room temperature for 30 min in dark place. At 517 nm, the absorbance was measured. The total RSA of each extract was expressed as the percentage of DPPH reduced and was calculated by the following equation:

$$\text{RSA\%} = \frac{A_0 - A}{A_0} \times 100,$$

A0, absorbance of DPPH solution without any antioxidant; A, absorbance of DPPH solution after reaction with the extract. All experiments were performed in triplicate.

Analysis of fatty acid composition

The EVOO sample was vigorously shaken in n-hexane (0.2 g in 3 ml n-hexane) with 0.4 ml of 2 M methanolic potassium hydroxide solution to extract fatty acids. Fatty acids (FAs) were coupled with on a gas chromatograph HP 7890 (II) and HP 5975 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with an electron impact ionization of 70 eV.

The temperature was set to climb from 40 to 280 °C at a rate of 5 °C/min using an HP-5MS capillary column (30m 0.25mm, 0.25m film thickness; Agilent Technologies, Hewlett-Packard, CA, USA). At a flow rate of 1.2 ml/min, a split ratio of 60:1, and scan times and mass ranges of 1 s and 40–300 m/z, respectively, helium was utilized as the carrier gas. The GC–MS data system's Wiley 09 NIST 2011 mass spectral library was used to compare the recorded mass spectra with those contained in the Wiley 09 NIST 2011 mass spectral library to identify FAs.

Phenolic compounds

Extraction of phenolic compounds

Liquid–liquid extraction was used for the extraction of phenolic chemicals [48]. In brief, 2 g of sample material was precisely weighed into a 10 ml glass tube, then 5 ml MeOH/H2O (80/20 (v/v) was added, the mixture was shaken for 1 min (Vortex, 1500 Mot/min), then centrifuged for 15 min at 3000 rpm.

The methanol collected was extracted with a Pasteur pipette after centrifugation, and the solvent was transferred to a flask. For a complete extraction of the oil, the extraction procedure was conducted a second time with the residual oil. The first and second extracts were blended. Finally, the combined extract's solvent was evaporated under nitrogen at 40 °C, and the residue was dissolved in 500 μliters of MeOH/H$_2$O (50/50 (v/v)). The dissolved extract was shaken

for 1 min (Vortex (1500 Mot/min), then filtered using a syringe filter (PTFE 0.2 m, WICOM Germany GmbH, Heppenheim, Germany), and then transferred to a vial for injection into the HPLC–DAD or HPLC–ESI–QToF–MS system [48].

HPLC–ESI–QToF–MS analysis

A HPLC UltiMate 3000 (Thermo Fisher Scientific, Sunnyvale, CA, USA) equipped with an autosampler was used to analyze the polar extract using a high-performance liquid chromatography–electrospray ionization–quadruple time of flight mass spectrometer (HPLC–ESI–QToF–MS). The samples were stored at 5 °C. A 150 mm X 2.1 mm, 1.7 m Kinetex column EVO C18 was used for separation at 40 °C (Phenomenex, Torrance, CA, USA).

The mobile phase was A = water with 0.01% HCOOH and B = MeOH with 0.01% HCOOH. In a total run period of 18 min, the percentage of organic modifier (B) was varied as follows: 0 min, 10%; 5 min, 30%; 14 min, 60%; 14.50 min, 70%; 14.51 min, 100 percent; 16.00 min, 100 percent; 16.01 min, 10%. The injection volume was 10 μ liters. Following negative electrospray ionization, the detection was performed on a Maxis Impact HD (Bruker Daltonik, Bremen, Germany) in MS/MS (broadband collision induced dissociation (bbCID) mode with a diode array detector at wavelengths of 235, 280, and 335 nm. Nitrogen was employed as a nebulizing and desolvation gas.

MS data were collected in the m/z range of 50–1000. The other parameters' values were determined as follows: 3000 V capillary voltage; 200 °C drying gas temperature; 8 L/min dry gas flow; 2 bar nebulizing gas pressure; 500 V plate offset. For the first 0.5 min of each run, a calibration solution of sodium formate (10 mM) was injected into the MS (flow: 0.18 L/min).

The data were collected using the Compass of Series 1.7 package as software. A blank MeOH/H2O (10/90 (v/v)) was used after every ten injections to check for contamination in the system [48].

Data analysis

The data from the HPLC–ESI–QToF were analyzed using Data Analysis (Bruker). MS spectra were used to determine the mass to charge ratio (m/z) of the molecular ion peak [M-H] of the suspect substances. Potential sum formulae were calculated using the software application "3D smart formula." A database of phenolic compounds called Phenols Explorer (INRA, Paris, France) was used to identify phenolic compounds that have the same formula.

The peaks produced by HPLC–DAD analysis were evaluated and integrated using EZ Chrom Elite. The components were identified by comparing the retention time and UV spectra of the analytical standards to those of the analytical standards, which were chosen based on the assumptions obtained from the HPLC–ESI–ToF–MS studies.

The samples were examined in triplicate for the quantitative analysis, and the findings were presented as mean ± standard deviation. External calibration was used to perform the quantification. The calibration was done using 4-hydroxy benzaldehyde, vanillin, syringaldehyde, *p*-coumaric acid, ferulic acid, and ferulaldehyde at concentrations of 0.3 mg/L, 1.0 mg/L, 2.5 mg/L, 5.0 mg/L, 10.0 mg/L, 15.0 mg/L, 25.0 mg/L, and 50.0 mg/L. A calibration curve made of *p*-coumaric acid was used to quantify the ethyl ester of *p*-coumaric acid [48].

Tocopherols

The HPLC was used to analyze tocopherols using a solution of 250 mg of oil in 25 ml of n-heptane. A Merck-Hitachi low-pressure gradient system with an L-6000 pump, a Merck-Hitachi F-1000 fluorescence spectrophotometer (detector wavelengths for excitation 295 nm, for emission 330 nm), and a D-2500 integration system were used to conduct the HPLC study. The samples were injected into a Diol phase HPLC column 25 cm 4.6 mmID (Merck, Darmstadt, Germany) with a flow rate of 1.3 ml/min using a Merck 655-A40 autosampler. n-heptane/tert-butyl methyl ether (99 + 1, v/v) was utilized as the mobile phase [11].

Statistical analysis

The results are reported as the mean values. Data of analytical characteristics of oils were analyzed by ANOVA/MANOVA using the XLSTAT 2014. Duncan's multiple range tests were used to determine significant differences among data.

Results and discussion

Quality parameters

Table 1 shows the physicochemical quality and antioxidant parameters of the studied oils. All the analyzed oils showed very low values for the regulated physicochemical parameters evaluated (acidity ˂0.8%; peroxide value ≤ 20 meq O$_2$/kg; K$_{270}$ ≤ 0.22; K$_{232}$ ≤ 2.5, and Δk ≤ 0.01), with all of them falling within the ranges established for ''extra virgin olive oil'' category, as required by IOOC Regulation (IOOC 2019). The K$_{232}$ index correspond to the absorbency of conjugated dienes: first oxidation products and the K$_{270}$ index correspond to the absorbency of the secondary oxidation products: conjugated trienes (Aggarwal et al. 2021).

Table 1 Quality characteristics of the studied enriched olive oils

Quality characteristics	Chemlali oil	Chemlali oil + 1% wild olive leaves	Chemlali oil + 3% wild olive leaves	Chemlali oil + 5% wild olive leaves	Chemlali oil + 10% wild olive leaves
Free fatty acid (% oleic)	0.3 ± 0.04^a	0.38 ± 0.01^a	0.3 ± 0.01^a	0.42 ± 0.02^a	0.41 ± 0.01^a
Peroxide value (meq/kg)	3.5 ± 0.04^{ab}	3.95 ± 0.01^{ab}	2.91 ± 0.01^b	3.97 ± 0.03^{ab}	4.8 ± 0.01^a
K_{232}	1.20 ± 0.04^a	1.32 ± 0.04^a	1.24 ± 0.02^e	1.35 ± 0.01^a	1.42 ± 0.01^a
K_{270}	0.12 ± 0.04^a	0.13 ± 0.02^a	0.1 ± 0.01^a	0.11 ± 0.01^a	0.11 ± 0.01^a
Δk	0.0039 ± 0.24	0.0039 ± 0.24	0.00215 ± 0.02	$0.002250.24 \pm$	0.00245 ± 0.24
Chlorophylls (mg/kg)	1.68 ± 0.04^b	1.90 ± 0.04^b	2.28 ± 0.02^b	2.54 ± 0.03^b	4.7 ± 0.01^a
Carotenoids (mg/kg)	1.02 ± 0.24^b	1.03 ± 0.24^b	1.12 ± 0.01^b	2.20 ± 0.24^b	7.17 ± 0.24^a
DPPH (%)	45.7 ± 0.12^d	46.6 ± 0.24^d	63.8 ± 0.36^c	72.2 ± 0.54^b	83 ± 0.24^a

Data are expressed as mean values of five independent experiments. Duncan's test has been used to assess significance (Duncan's test, $P < 0.05$). Values followed by different letters ([a,b,c,d] and [e]) in the same line are significantly different. DPPH: radical-scavenging activity. All experiments were performed in triplicate

Note that lower values for these parameters will translate into a higher quality of the oil obtained from fresh and healthy olives, harvested at the optimal ripening point, followed by immediate extraction without proceeding to olive storage (Aggarwal et al. 2021). It is known that olives at later ripeness stages give oils with higher levels of free acidity since they undergo an increase in enzymatic activity, especially lipolytic enzymes, and are more sensitive to mechanical damage and pathogenic infections [7, 31].

The addition of leaves via maceration process did not affect the quality parameters of the fortified olive oils. It has been reported that, the addition of leaves during oil extraction and processing prevented the oxidation and the formation of peroxides, reducing the peroxide value to half [27, 30]. Contrary to our results, Malheiro et al. [32] reported that leaf addition increased the peroxide value. Such discrepancy could be explained with differences in the relative presence of additional antioxidants of leaf residues. These compounds may have enhanced the oxygen availability through gas exchanges that occur during the respiration process, resulting in peroxidation [42, 43].

Chlorophylls and carotenoids

Olive oils contain a lot of chlorophyll and carotenoids [12]. They work as antioxidants in the dark and as prooxidants when exposed to light, interfering with oxidative stability [30]. Furthermore, these chemicals are responsible for the olive oil's yellow-green color, which increases customer acceptance. Table 1 shows the levels of chlorophyll and carotenoids in studied olive oils. In the present study, the adding of wild olive leaves (1–3–5 and 10%) to Chemlali oils enhanced chlorophyll and carotenoid concentrations according to maceration percentage. The Chemlali enriched oil showed a higher chlorophyll content (Table 1) in comparison with Chemlali control. In addition, EVOO from

Chemlali cultivar extracted with 10% added of wild olive leaves presented the highest amount of chlorophyll (4.7 mg/kg) and carotenoid (7.17 mg/kg) (Table 1) contents.

The addition of wild leaves also made the olive oils seem greener, which was seen in all oils. Because of the antioxidant activity of chlorophyll and its ability to conduct chemopreventive activities against carcinogens, the fortified oils become nutritionally attractive as their chlorophyll concentrations rise [30], [42].

Radical-scavenging activity (RSA)

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a colorless hydrazine that may absorb labile hydrogen atoms from phenolic antioxidants (DPPH-H). The free radical-scavenging activity (RSA) of an extract can be expressed as the percentage of DPPH reduced by a given amount of extract [30]. Due to the harmful effects of free radicals in foods and biological systems, radical-scavenging activity is critical [42]. This test is a typical assay in antioxidant activity investigations and provides a quick method for determining a compound's radical-scavenging activity [42]. The stable free DPPH radical is a helpful reagent for studying bioactive compounds' scavenging activities. The radical-scavenging capacity of the examined samples differed significantly: olive oil enriched with 10% of leaves added had the greatest radical-scavenging activity (83%), while for the rest of the samples, their percentages were below 80%, ranging between 45.7 and 72.2% (Table 1) highlighting the role that the addition of wild olive leaves could have had in the olive oil oxidative stability [32, 46].

Fatty acid profile

The fatty acids composition of the olive oils extracted with different percentages of olive leaves was analyzed and the respective profiles are given in Table 2. The fatty

Table 2 Fatty acid compositions of the studied enriched olive oils

Quality characteristics	Chemlali oil	Chemlali oil + 1% wild olive leaves	Chemlali oil + 3% wild olive leaves	Chemlali oil + 5% wild olive leaves	Chemlali oil + 10% wild olive leaves
Palmitic C16:0%	16.43 ± 0.04^a	16.87 ± 0.04^a	17.23 ± 0.02^a	17.07 ± 0.02^a	16.86 ± 0.03^a
Palmitoleic C16:1%	2.4 ± 0.04^a	2.22 ± 0.01^a	2.32 ± 0.02^a	2.21 ± 0.02^a	$2.11 \pm 0.O3^a$
Stearic C18:0%	2.19 ± 0.04^a	2.37 ± 0.01^a	2.42 ± 0.03^a	2.43 ± 0.02^a	2.46 ± 0.02^a
Oleic C18:1%	62.35 ± 0.4^a	61.03 ± 0.24^a	61.05 ± 0.54^a	60.9 ± 0.64^a	61.53 ± 0.2^a
Linoleic C18:2%	15.94 ± 0.04^a	15.55 ± 0.04^a	15.12 ± 0.04^a	15.41 ± 0.8^a	15.27 ± 0.1^a
Linolenic C18:3%	0.09 ± 0.24^a	0.24 ± 0.24^a	0.16 ± 0.24^a	0.24 ± 0.24^a	0.19 ± 0.24^a
Arachidic C20:0%	1 ± 0.04^a	1.06 ± 0.01^a	1.06 ± 0.02^a	1.10 ± 0.01^a	1.06 ± 0.01^a
MUFAs%	64.75 ± 0.2^a	63.25 ± 0.24^a	63.37 ± 0.24^a	63.11 ± 0.24^a	63.64 ± 0.24^a
PUFAs%	16.03 ± 0.12^a	15.79 ± 0.36^a	15.28 ± 0.24^a	15.65 ± 0.44^a	15.46 ± 0.36^a
C18.1/ C18.2	4.03 ± 0.04^a	4 ± 0.03^a	4.14 ± 0.02^a	4.03 ± 0.01^a	4.11 ± 0.05^a

Data are expressed as mean values of five independent experiments. Duncan's test has been used to assess significance (Duncan's test $P < 0.05$). MUFAs: monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids. Values represent means ± standard deviations. Different lower case letters in the same row indicate significantly different values per $p < 0.05$
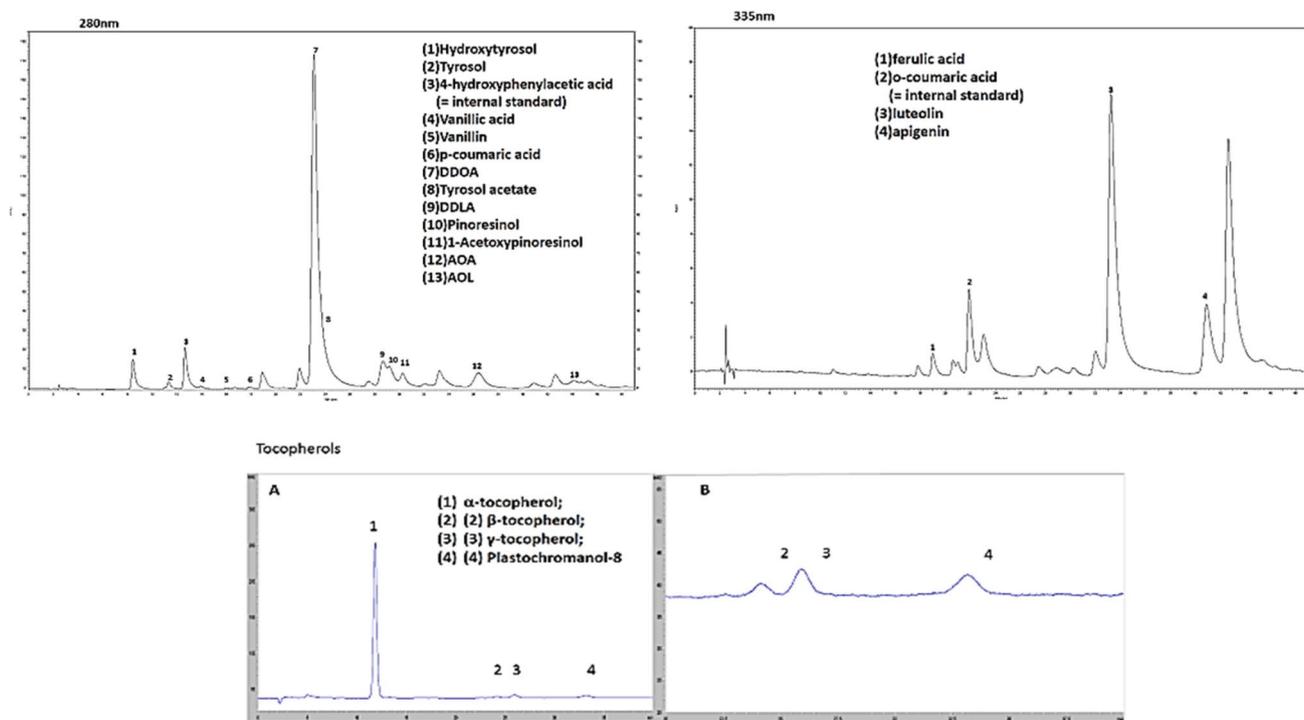

Fig. 1 Chromatograms of phenols and tocopherols detected in enriched olive oils (+10% wild olive leaves) separated by LC/MS/QTOF and HPLC–FLD. A: 0–40 min; B: 20–40 min

acid profiles obtained in all samples are in accordance with those regulated for olive oil (IOOC 2019). As expected, the most abundant fatty acid in all samples was (Fig. 1) the oleic acid (C18:1), followed by palmitic acid (C16:0) and linoleic acid (C18:2) independently of the percentage of olive leaves added. The content of the three main fatty acids did not follow any tendency with the increasing quantity of olive leaves added. Moreover, monounsaturated fatty acid (MUFA) showed the same tendency as oleic acid is the

major responsible for MUFA content. Similarly, the oleic/linoleic acid ratio varies from 4 to 4.11 for all studied oils (Table 2).

Tocopherols

Tocols are naturally present in oils and play an important role in their resistance to oxidation processes. Table 3 describes the changes on the tocopherols, tocotrienols, and

Table 3 Tocopherol compositions of the olive oil samples (results are expressed as mg kg⁻¹ and percentage of total tocopherols)

Samples	α-T	α-T3	β-T	β-T3	γ-T	γ-T3	δ-T	δ-T3	P8	Total (mg kg⁻¹)
Chemlali oil	208 .36±1.54[c]	0.00	1.45±0.11[c]	0.00	4.63±0.30[e]	0.00	0.00	0.00	2.76±0.29[e]	217.20±5.26[b]
Chemlali oil + 1% wild olive leaves	238.40±1.47[b]	0.00	2.79±0.38[a]	0.00	6.07±0.23[a]	0.00	0.00	0.00	6.86±0.51[a]	254.13±15.54[a]
Chemlali oil + 3% wild olive leaves	250.84±18.35[a]	0.00	2.41±0.05[a]	0.00	6.14±0.41[a]	0.00	0.00	0.00	5.59±0.20[b]	264.99±14.92[a]
Chemlali oil + 5% wild olive leaves	237.42±1.60[b]	0.00	2.61±0.02[a]	0.00	5.63±0.27[b]	0.00	0.00	0.00	4.48±0.25[d]	250.13±5.10[a]
Chemlali oil + 10% wild olive leaves	231.45±1.86[b]	0.00	2.05±0.12[b]	0.00	5.19±0.26[b]	0.00	0.00	0.00	5.24±0.12[c]	243.92±16.33[a]

α-T α-tocopherol; *β-T* β-tocopherol; *γ-T* γ-tocopherol; *δ-T* δ-tocopherol; P8: plastochromanol-8 *α-T3* α-tocotrienol; *β-T3* β-tocotrienol; *δ-T3* δ-tocotrienol; *Total T* total tocopherols. Values represent means ± standard deviations. Different lower case letters in the same row indicate significantly different values per $p < 0.05$

plastochromanol-8 contents in olive oils macerated with wild olive leaves. Three tocopherols (α-, β-, and γ-tocopherol) and one plastochromanol-8 were identified and quantified, tocotrienols were not detected in all studied oils. α-Tocopherol being the most abundant in all studied olive oils (Figs. 2 and 3). It can be seen in Table 3 that the highest tocopherol contents were obtained in fortified oil. This result is related mainly with the α-tocopherol content. In fact, some authors (Lucas et al. 2002) considered olive leaves as an alternative source of α-tocopherol.

The content of α-tocopherol was increased showing statistically significant differences between the olive oils without leaves and those up to 1% of leaves added (Table 3), raising the content of α-tocopherol by about 20% in comparison with the control (Fig. 2).

Alpha tocopherol accounts for about 90% of the total tocopherols in olive oil (Aggarwal et al. 2021). Tocopherol content is very important for oxidative stability of olive oil because of its ability to scavenge peroxyl radicals [4]. Significant augmentation in α-tocopherol contents ($P < 0.05$) were observed in the enriched oils according to the proportions of macerated leaves, ranging between 208.36 and 250.84 mg/kg.

Significant increased in β-tocopherol contents ($P < 0.05$) were also observed in the samples, ranging from 1.45 and 2.79 mg/kg. Similarly, an increases was observed ranging between 12 and 32.6% of plastochromanol-8 (P8) contents than the control one with the addition of leaf material up to 1% level. Plastochromanol-8 is a natural lipophilic antioxidant found in vegetable oils [6, 20]. Similarly to β-tocopherol, the content of plastochromanol-8 was the highest in olive oil with 1% (Table 3), when it strongly increased with leaves addition during maceration (Table 3), Plastochromanol-8 is considered to be a natural homologue of γ-tocotrienol that contains a longer side chain [12], [42].

Baškirovset al. [12] also studied the antioxidative characteristics of plastochromanol-8 and found that it was a better natural antioxidant than α-tocopherol in the prevention of autoxidation.

In the present study, leaf addition significantly increased tocopherol content in all samples, therefore, leaf-added oils could have more functional properties compared to control samples. Malheiro et al. [32] revealed approximately 30% increase in α-tocopherol amount with the addition of 10% of fresh wild leaves. This is the first report showing the presence of an array of tocopherol compounds in olive oil enhanced with oleasters olive leaves. The increase of tocopherols and plastochromanol-8 content in the flavored oils could be related to the tocopherol compounds migration from oleaster leaves to oil. In fact, wild olive leaves species were reported to be rich sources of tocopherol compounds [7]. Additionally, wild olive oil is described as rich in antioxidant compounds [6], [9].

Fig. 2 Tocopherol contents of the studied olive oils. α-T: α-tocopherol; β-T: β-tocopherol; γ-T: γ-tocopherol; δ-T: δ-tocopherol; P8: plastochromanol-8; α-T3: α-tocotrienol; β-T3: β-tocotrienol; δ-T3: δ-tocotrienol
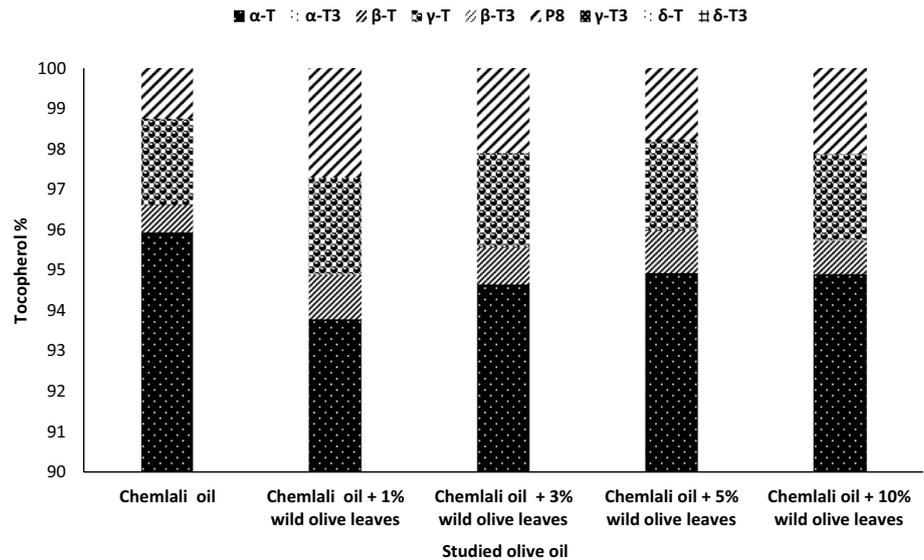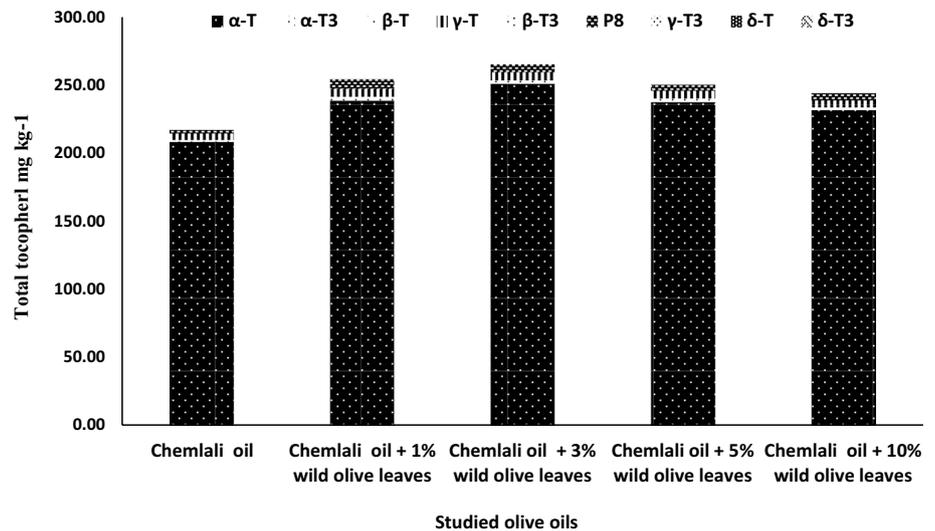

Fig. 3 Percentages of different tocopherol compounds of the studied enriched olive oils. α-T: α-Tocopherol; β-T: β-Tocopherol; γ-T: γ-Tocopherol; δ-T: δ-Tocopherol; P8: Plastochromanol-8; α-T3: α-Tocotrienol; β-T3: β-Tocotrienol; δ-T3: δ-Tocotrienol

Besides being an important aspect due to its natural antioxidant activity, from a nutritional point of view this increase allows a greater availability of vitamin E, increasing olive oil health benefits and prevention of deficiency symptoms [42].

Tocopherols are reported to protect oils against oxidation through two basic mechanisms: a chain-breaking electron donor mechanism and a chain-breaking acceptor mechanism (Azzi et al. 2019; [12]. According to Lorini et al. [30], antioxidant molecules can extend the shelf life of oils by reducing the lipid peroxidation process, consequently, alternative natural and safe sources of dietary antioxidants are needed. The findings demonstrate that this purpose was achieved by combining leaves with olive oil before extracting the oil [42].

Phenolic compounds

The presence of phenolic compounds is one of the reasons for the nutritional value and shelf life of virgin olive oil. The most common phenolic chemicals in studied EVOO are secoiridoids (Figs. 4 and 5), which are oleuropein derivatives (Garca et al. 2015). The phenolic components in the enriched EVOOs were studied using LC–ESI–TOF/MS. All of the olive oils examined had low quantities of flavonoids and high levels of secoiridoid compounds and derivatives. The phenolic profile of the enhanced oils was very comparable. There were quantitative differences, particularly in the proportion of the secoiridoids. Based on infusion percentages, all of these components might be used as a fingerprint to identify and differentiate distinct olive oils [19], [48]. The amounts of total phenols show significant

Fig. 4 Identification contents of different phenolic classes of the studied enriched olive oils
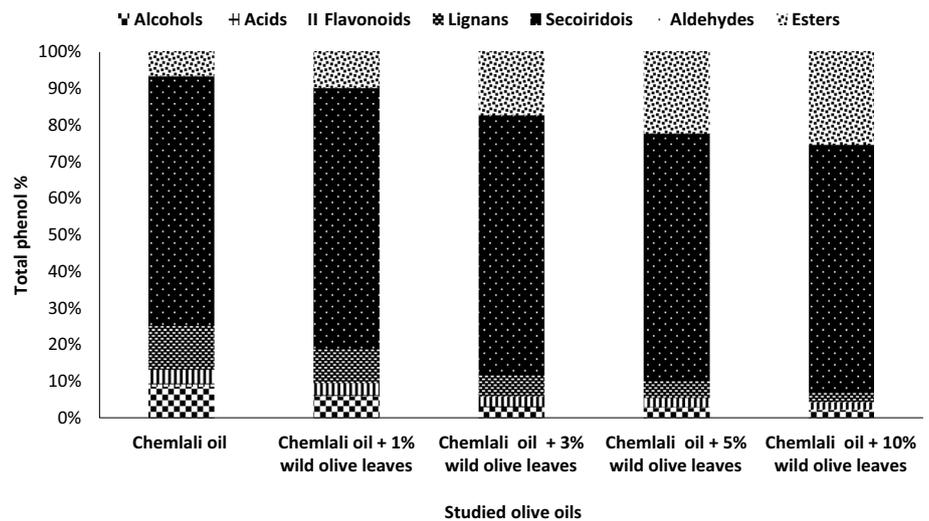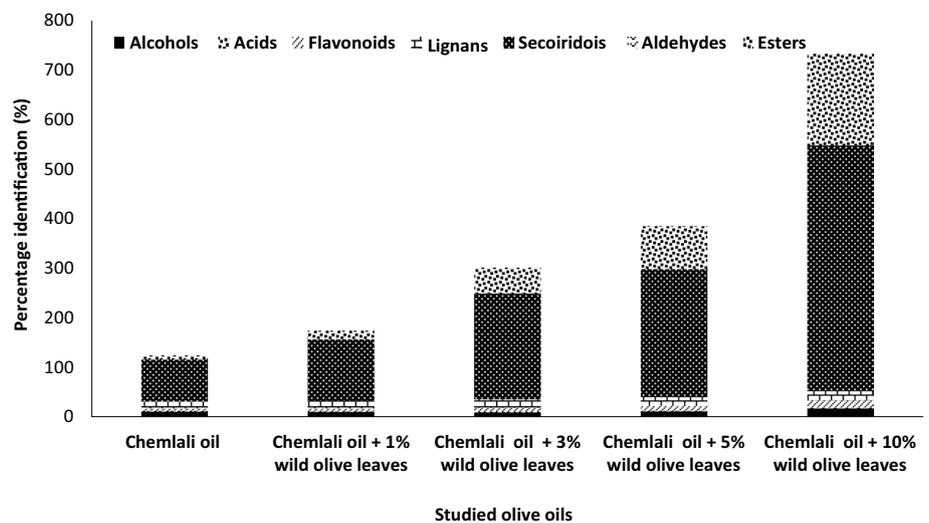

Fig. 5 Distribution percentages of different phenolic groups of the studied enriched olive oils

differences ($P < 0.05$) among the different proportion of wild olive leaves addition (Table 4). Nevertheless, a significant increase was observed in the olive oils with 10% of leaves for phenols ($P < 0.05$).

There were significant differences ($p < 0.05$) between the various enriched oils. Our findings are in line with previous research, which indicates that the total phenolic content of olive oils varies from 50 to 1000 mg kg^{-1} [8], [41]. Fifteen compounds from different families were identified. Quantitative data for the phenolic are reported in Table 4. Seven main phenolic groups were detected: phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoids (mainly derivatives of oleuropein and ligstroside), lignans (Pinoresinol, 1-Acetoxypin), flavonoids (luteolin and apigenin), and phenolic acids (Vanillic acid, p-Coumaric acid, Ferulic acid), aldehyde (vanillin), ester (tyrosyl acetate).

Foods colors and sensory attributes, as well as its health-related antioxidant effects acids have all been linked to phenolics [35]. All the studied EVOOs showed low concentration of this class of compounds and did not make important variations between maceration percentages, as the content ranged from 0.82 to 1.1 mg kg^{-1}. Vanillic acid was found in all samples analyzed with a content that varied between 0.35 and 0.56 mg kg^{-1} for control and 10% leaf-added oils, respectively. Although, P-coumaric acid concentration on the contrary was decreased according to leaf additions. The third acid compound detected is ferulic. The last was found at low concentrations and did not make important variations between maceration percentages.

Hydroxytyrosol and tyrosol, both generated from the hydrolysis of oleuropein aglycon and ligstroside aglycon, respectively, were the main phenolic alcohols in the studied olive oils [30], [35]. Similarly, we observed increases

Table 4 Phenolic compounds compositions of the studied enriched olive oils

Phenolic compounds	Chemlali oil	Chemlali oil + 1% wild olive leaves	Chemlali + 3% wild olive leaves	Chemlali + 5% wild olive leaves	Chemlali + 10% wild olive leaves
Hydroxy tyrosol	5 2 ± 0,24[a]	6.39 ± 0,18[a]	5.38 ± 0.12[a]	6.93 ± 0.08[a]	12.93 ± 0.77[a]
Tyrosol	5.64 ± 0.2[a]	4.01 ± 0.2[a]	3.86 ± 0.24[a]	4.15 ± 0.24[a]	4.33 ± 0.24[a]
Vanillic acid	0.35 ± [a]	0.43 ± 0.2[a]	0.45 ± 0.2[a]	0 .47 ± 0.24[a]	0.56 ± 0.24[a]
Vanillin	0.09 ± 0.2[a]	0.07 ± 0.2[a]	0.09 ± 0.2[a]	0.1 ± 0.24[a]	0.1 ± 0.24[a]
p-Coumaric acid	0.63 ± 0.2[a]	0.22 ± 0.2[a]	0.17 ± 0.2[a]	0.25 ± 0.24[a]	0.19 ± 0.24[a]
DDOA	9.67 ± 0.2[a]	35.03 ± 0.2[a]	117.31 ± 0.[a]2[a]	152.39 ± 0.24[a]	366.8 ± 0.24[a]
Tyrosyl acetate	8.36 ± 0.2[a]	17.36 ± 0.2[a]	52.36 ± 0.2[a]	86.13 ± 0.24[a]	186.82 ± 0.24[a]
DDLA	19.33 ± 0.2[a]	26.02 ± 0.2[a]	35.55 ± 0.2[a]	42.41 ± 0.24[a]	60.04 ± 0.24[a]
Pinoresinol	3.06 ± 0.2[a]	2.83 ± 0.2[a]	3.81 ± 0.2[a]	4.07 ± 0.24[a]	5.3 ± 0.24[a]
1-Acetoxypin	11.84 ± 0.2[a]	13.57 ± 0.2[a]	13.48 ± 0.2[a]	14.12 ± 0.24[a]	13.8 ± 0.24[a]
AOA	37.79 ± 0.2[a]	48.34 ± 0.2[a]	47.56 ± 0.2[a]	51.61 ± 0.24[a]	53.44 ± 0.24[a]
AOL	17,63 ± 0.2[a]	14 ± 0.2[a]	12.55 ± 0.2[a]	11.47 ± 0.24[a]	14,04 ± 0.24[a]
Ferulic acid	0.12 ± 0.2[a]	0.17 ± 0.2[a]	0.25 ± 0.2[a]	0.33 ± 0.24[a]	0.34 ± 0.24[a]
Luteolin	2.9 ± 0.2[a]	3.18 ± 0.2[a]	5.67 ± 0.2[a]	7.26 ± 0.24[a]	11.84 ± 0.24[a]
Apigenin	1.7 ± 0.2[a]	2.37 ± 0.2[a]	2.29 ± 0.2[a]	2.32 ± 0.24[a]	2.76 ± 0.24[a]
Total phenol (mg/kg)	124.33 ± 0.2[a]	174.02 ± 0.2[a]	300.78 ± 0.2[a]	384.01 ± 0.24[a]	733.28 ± 0.5[a]

DDOA dialdehydic form of decarboxymethyl oleuropein aglycone; *DDLA* dialdehydic form of decarboxymethyl ligstroside aglycone; *AOA* aldehydic form of oleuropein aglycone; *AOL* aldehydic form of ligstroside aglycone; 1-Acetoxypin: 1-acetoxypinoresinol. Values represent means ± standard deviations. Different lower case letters in the same row indicate significantly different values per $p < 0.05$

of, hydroxytyrosol. With regard to their levels, hydroxytyrosol, the most active antioxidant phenolic compound found at high concentration in olive oils with the addition of leaf material at the 10% level exceeding 12.9 mg kg^{-1} as compared to control oil.

The content of tyrosol through the four maceration levels is different from that for the control oil. Thus, the tyrosol concentration decreases in enriched oils through the maceration process. This increment agrees with the results of previous studies [30], [35].

The concentration of tyrosol in control oil was higher than that of hydroxytyrosol. These results are similar to those reported by several authors [31, 44]. In contrast, tyrosol contents were relatively higher than those of hydroxytyrosol for the other enriched olive oils, which is in accordance with results revealed by some authors [37], [41].

Numerous studies have evaluated the antioxidant capability of hydroxytyrosol for its prevention role against tumoral and cardiovascular diseases [38] (Marrone et al., 2020). On the other hand, Warleta et al. [47] signaled that the simple phenol hydroxytyrosol could contribute to the preventive cancer activity attributed to VOOs, due to the reduction of oxidative stress and oxidative DNA protection in normal breast cells at physiological concentrations. Moreover, the European Food Safety Authority [23] has recently claimed that 'the consumption of olive oil rich in polyphenols (hydroxytyrosol, 5 mg/day) contributes to the protection of blood lipids from oxidative damage.

A marker useful for the varietal characterization of the studied olive oils is the presence of the tyrosyl acetate compound. In comparison with the control sample, significant increase in tyrosyl acetate compound ($P < 0.05$) was also observed in the samples enriched by wild olive leaves. The addition of increasing amounts of wild olive leaves material to oils significantly increased tyrosyl acetate compound from 8.36 mg kg^{-1} (0% addition) to 17.36 mg kg^{-1} (1% addition), 52.36 mg kg^{-1} (3% addition), 86.13 mg kg^{-1} (5% addition), and 186.13 mg kg^{-1} (10% addition).

On another front, lipophilic derivatives of tyrosol and, in particular, esters bearing acyl chains, exhibit an enhanced affinity with lipophilic membrane constituents [15, 18]. For this reason and others, these compounds could be important for further application in pharmaceutical and cosmetic fields [15, 18]. Some tyrosyl derivatives have been found in diverse natural sources, e.g., the presence of its acetate was reported in virgin olive oil [16]. Furthermore, in recent years, the utilization of several natural tyrosyl esters for antiaging and/or pharmacological uses has piqued attention [37], [41].

On the other hand, aldehyde class was represented exclusively by vanillin. This compound was also found in all samples and did not vary significantly according to the addition of leaves, ranging from 0.09 to 0.1 mg kg^{-1}.

Regarding secoiridoids, oleuropein, and ligstroside aglycones together with their derivatives were detected as shown in Table 4. In all analyzed olive oil samples, secoiridoids were by far the most abundant group of phenolic compounds. This dominance of secoiridoids in the phenolic fraction of

the control oil is also reported in literature [18], [45]. On the one hand, the addition of increasing amounts of leaf material to oils significantly increased secoiridoids amounts from 84.42 mg kg^{-1} (0% addition) to 494.32 mg kg^{-1} (10% addition).

It can be seen in Table 4 that, the effect of leaf addition on the quantitative content of secoiridoids can be clearly observed with the variation of the concentration of the predominant compounds in this group. AOA (C$_{19}$H$_{24}$O$_8$—Secoiridoid), the aldehydic form of oleuropein aglycone was the most abundant complex phenol in control Chemlali oil (37.79 mg kg^{-1}). Adding leaves (w/w) to oil increased drastically the AOA secoiridoid content by 25–40% (Fig. 4).

In addition, notable variations have been observed in the content of other determined secoiridoids derivatives; three main compounds were identified at relatively high concentrations. The first one was the dialdehydic form of decarboxymethyl ligstroside aglycone (DDLA, C$_{17}$H$_{20}$O$_5$—Secoiridoid), the second most abundant complex phenol in control Chemlali oil (19.33 mg kg^{-1}), increases to reach 60.04 mg kg^{-1} (10% leaf-added oils).

The second one was AOL, the aldehydic form of ligstroside aglycone (C$_{19}$H$_{24}$O$_7$_Secoiridoid), with contents ranging from 11.84 to 14.12 mg kg^{-1} for control and 5% leaf addition oils, respectively. The content of AOs reduced with leaf addition as compared to control oil.

The last one, the dialdehydic form of decarboxymethyl oleuropein aglycone (DDOA, C$_{17}$H$_{20}$O$_5$—Secoiridoid), was drastically augmented in enriched olive oils, the content increases from 9.67 mg kg^{-1} to 366.8 mg kg^{-1} with the addition of 10% of wild fresh leaves.

In terms of lignans, Loubiri et al. [31] identified pinoresinol and acetoxypinoresinol as the most common lignans in VOO. Two lignans, pinoresinol and acetoxypinoresinol, were identified in this study. Lignans contents varied between 14.9 mg kg^{-1} and 19.1 mg kg^{-1} for control and 10% added leaf oils, respectively.

Pinoresinol was present at concentrations ranging from 3.06 mg kg^{-1} in control oil to 5.3 mg kg^{-1} in 10% added leaf oils. Acetoxypinoresinol content in studied oils varied from 11.84 to 14.2 mg kg^{-1}, being more abundant than pinoresinol. These findings are consistent with those published by Becerra et al. (2018) [13] and Bajoub et al. [10], but differ from those obtained by Krichene et al. [28] for Arbequina oils, where acetoxypinoresinol content was found to be very low in contrast to pinoresinol.

The low amount of acetoxypinoresinol has recently been presented as a methodology for oil authenticity [31]. However, none of these lignans were discovered in olive oils from Neb Jmal and Picholine cultivars introduced in Tunisia, contradicting prior research [49] , [5].

These chemicals compounds, on the other hand, are not indicative of the cultivar variety, but rather of the olive oil extraction technique [31]. As Aranda et al. (2004) pointed out, these chemicals are the principal components of the phenolic fraction of the olive seed, and they are practically absent from the pulp, leaves, and limbs, therefore, their presence in the oil must be due to the pits shattering when the olives are crushed. These might be used as a measure of the crushing conditions and the ratio of fruit pulp to seed during olive processing.

As far as flavonoids are concerned, luteolin and apigenin were the two most common chemicals detected in all of the samples examined. This flavonoids has previously been found in olive cultivars grown in Tunisia, Spain, and Italy [31], [39], [7], [45]. The most prevalent flavonoid detected in the examined EVOOs was luteolin, which decrease according to leaf addition percentages. The content increases progressively from 2.9 to 11.84 mg/kg with the addition of leaf material. In addition, Apigenin was found in lesser concentrations, ranging from 1.7 to 2.76 mg kg^{-1} in control (0% addition) and enriched oils (10%), respectively.

Loubiri et al. (2019) reported that the degradation of oils was related to the antioxidant activity, fresh oils were 3–5 times more efficient than aged oils. When the interaction between storage period and the percentage of leaf addition is considered, the antioxidant activity decreased significantly in studied oils. However, at the end of 18 months of storage, the highest antioxidant activity was found in olive oils with 3% added leaf material. Moreover, Tarchoune et al. [42] reported that olive leaves can be considered as a potential natural antioxidant to prolong the shelf life of food products. The addition of olive leaves considerably increased total phenolic content in accordance with the results of Max et al. (2020). Ammar et al. [2] suggested that oils rich in phenols can be produced by adding olive leaf extract. Instead of olive leaf extract, olive leaves can also be added to fruits directly during crushing to increase the total phenolic content of oils [42].

In this context, total phenolic and tocopherol contents of olive oils are very important for the oxidative stability, and as reported in literature, a significant correlation is found between these parameters. It is reported that, the contribution of phenolic contents to Rancimat stability is nearly 51%, that of the fatty acid composition 24% and that of α-tocopherol 11% [3]. The use of olive leaf extract to increase the oxidative stability and antioxidant activity of olive oils has been a prominent theme in the literature [21], [29].

The inclusion of useful compounds recovered from olive by-products into nutritious meals is among the major benefits [42]. The usage of olive by-products in human nutrition has been shown to have a direct influence on health claims. Due to their antioxidant characteristics, several food companies have employed the phenolic portion of olive by-products, which mostly contains Hydroytyrosol and Oleuropein,

Fig. 6 Dendrogram of all collected data obtained using Euclidean distance. Chem: Chemlali; wol: wild olive oil
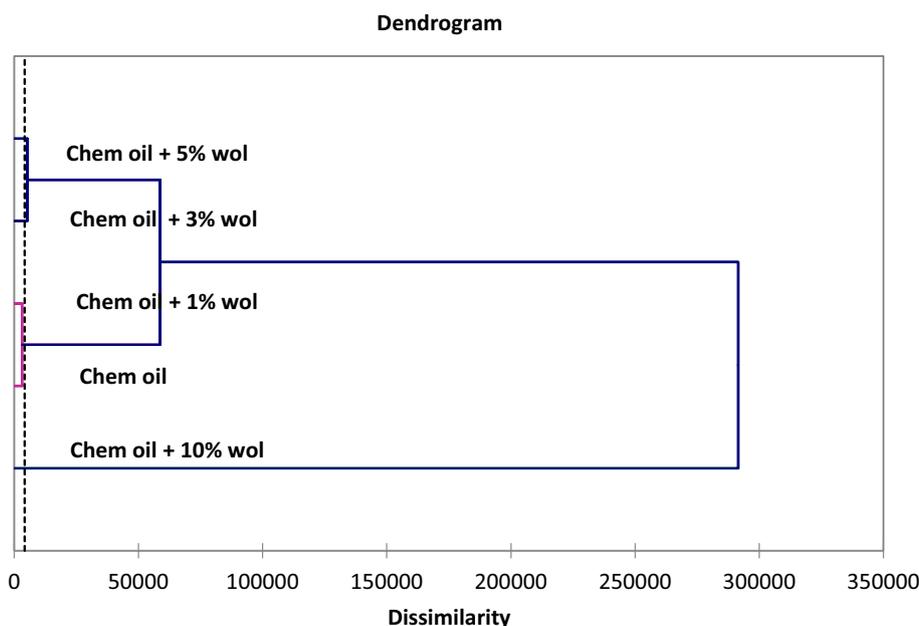
as food additives and preservatives [2]. Phenols and tocopherols found in olive oil, olive fruit, and leaf are considered to be responsible for these actions. Furthermore, using olive by-products to create new food and nutraceutical items is a novel strategy that fits present and future customer expectations for environmental impact, ethical concerns, human health, and safety (Max et al. 2020).

In addition, the amount of total phenols varies according to numerous parameters such as cultivar, climate, location, degree of ripeness, type of crushing machine, and oil process extraction, according to multiple authors [30], [40], [41].

Statistical analysis

All collected data were submitted to hierarchical cluster analysis to distinguish the five studied oils. This technique produces a hierarchy of partitions of objects such that any cluster of a partition is fully included in one of the clusters of the later partitions. Such partitions are best represented by
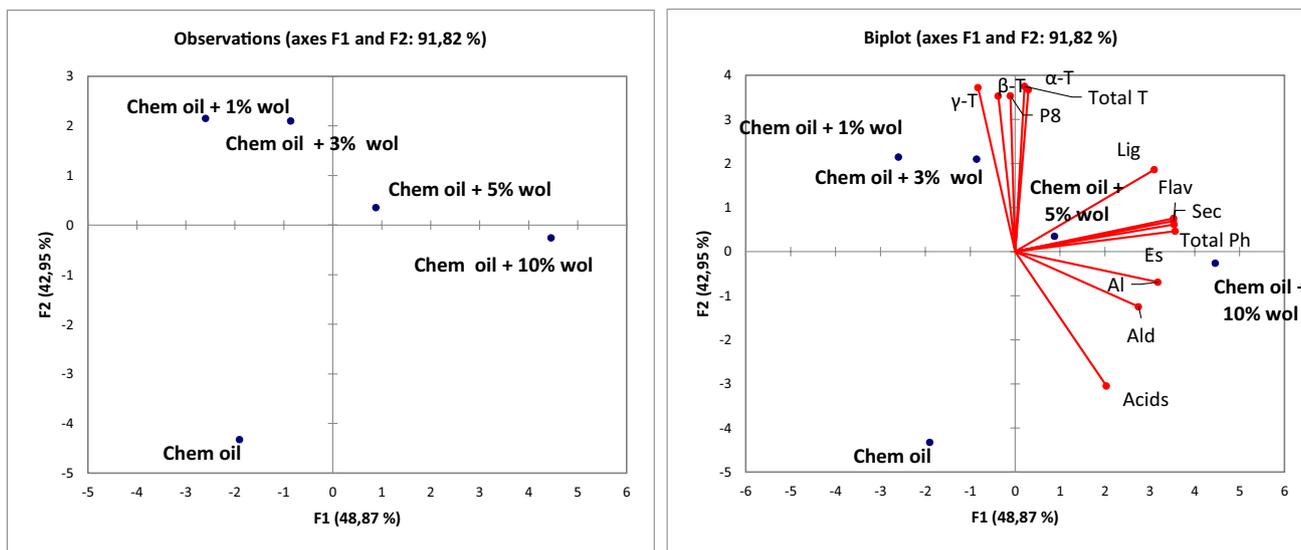

Fig. 7 PCA plot of the studied olive oils using the whole data set obtained. Chem: Chemlali; wol: wild olive oil; Lig: lignans; Ald: aldehydes; Al: alcohols; Total phe: total phenols; Es: esters; Flav: fla-vonoids; Sec: secoiridoids; α-T: α-tocopherol; β-T: β-tocopherol; γ-T: γ-tocopherol; Total T: total tocopherol; P8: plastochromanol-8

a dendrogram. The results as a dendrogram in Fig. 6, which indicates that the samples are distributed in four major clusters: the first group is constituted exclusively by control oil which is distinguished from the others for its low mean values of phenols and tocopherols contents. Control oil showed the lowest similarity to all studied varieties and was set apart from the two clusters.

The second group is formed by enriched oils with 3% added leaves. Cluster 3 is composed by oil with 5% added leaves, and finally Cluster 4 is constituted by oil enriched with 10% leaves addition.

To study how the studied parameters are useful in chemometric analysis to discriminate between oils, a principal component analysis (PCA) was performed. The first and the second principal components were sufficient to display the data structure, since they explained 91.82% of the total variance. By examining the scores-plot (Fig. 7) in the area defined by the first and the second principal components, the samples were separated into three groups based on bioactive compounds.

Group I is situated on the bottom left of the scores-plot and correlates negatively to both PC1 and PC2 and it is formed by Chemlali oil without leaves addition (control). Group II, is situated in the upper right side of the scores-plot and correlated positively to PC1 and PC2 include the oils enriched with 1 and 3% of wild olive leaves. Such a group is characterized by high content of tocopherol compounds. Group III, which is located on the left side of the scores-plot, is composed of oils enriched with 5% and 10% leaves. This group is distinguished especially by the high phenolic compounds contents.

These statistical analyses (PCA and HCA) can explain the variability of the oil composition according to the proportion of leaves addition. We note a good discrimination between different maceration concentrations according to phenol and tocopherol data. These components seem to be an effective tool to discriminate between the studied oils. It is evident the effect of maceration process.

Conclusions

This is the first report showing the presence of an array of bioactive compounds in olive oil enriched with wild olive leaves. The increases of phenolic and tocol contents in the enriched oil could be related to the antioxidant compounds migration from oleaster leaf to oil. In fact, *Olea europaea* L. subsp. sylvestris leaves were reported to be rich sources of phenolic compounds.

The information collected in this work shows that the by-products derived from the wild olive trees are secondary but valuable products, from which different biologically active molecules can be recovered by green extraction technologies and can be reused for food, pharmaceutical and cosmetic purposes following the circular economy policies.

Declarations

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