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ORIGINAL PAPER

Exploring the quality and nutritional profiles of monovarietal oils from millennial olive trees in Tunisia

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Abstract

In Tunisia, the olive tree has been cultivated for more than 3000 years by different civilizations, such as the Phoenicians, Greeks, Carthaginians, Romans, and Arabs. The ancient olive trees, which thrive in harsh environments, are little known and contain a foremost part of the olive germplasm. This study focused on the chemical oil analysis of the Tunisian millennial olive trees dating from the Roman and the Carthaginians periods. Twenty-eight antique olive trees grown in Tunisia's north to south are the subject of this study. Analyzed according to international standards, the tested accessions present oil with high quality, rich in chlorophyll and carotenes, and highly rich in polyphenols. The oil's fatty acid composition is complete with the international norms with a high oleic acid content, low palmitic and linoleic acid concentrations, and a C18:1/C18:2 ratio ≥ 7. Four oils, in particular, contained substantial levels of polyphenols (500–1632 mg/kg) and a C18:1/C18:2 ratio of >9. Subsequently, it is crucial to prevent the extinction of ancient olive trees to preserve their historical significance and ecological worth, as well as to incorporate the best genotypes into new varieties and boost the competitiveness of Tunisian olive oil on the global market.

Keywords Millennial olive trees · Oil quality · Polyphenols · Carotenes · Chlorophyll · Acid composition

Introduction

Olive oil is a foodstuff steeped in myths and cultures, with many important properties related to its organoleptic characteristics, nutritional value, and cultural influences. The development of olive cultivation in the Mediterranean is closely linked to human nutrition and, together with wheat and grapes, forms the "Mediterranean trilogy" on which the diet of the same name is based, widely recognized for its health benefits [1].

Extra virgin olive oil (EVOO) is defined as oil obtained directly from fresh fruits through a mechanical process without refining steps, which play an important role in the final quality of EVOO [2]. It is a high-quality nutrient due to its content of fatty acids, essentially unsaturated, and its minor components, such as polyphenols, tocopherols, and carotenoids [3]. These give it a high antioxidant activity that can help prevent or delay the onset of certain degenerative and cardiovascular diseases [4, 5].

One of the olive-growing countries that attract more and more attention for its high-quality oil is Tunisia, a North African country covered with olive groves and where olive oil presents an essential part of gastronomy. In this country, olive cultivation dates back to the Berbers, long before the arrival of the Carthaginians. Since then, the dietary habits of Tunisia's indigenous population have been based on olive fruit and oil, used by rural women to prepare bread, soup, porridge, and many other dishes [6]. Throughout history, olive oil has also been used to treat diseases, such as

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rheumatic and cardiovascular diseases, stomach ailments, and burns [7], which is why olive oil can be considered an integral part of Tunisia's history and the culture of its inhabitants. The centuries-old tradition of olive cultivation has given rise to today's olive groves which cover an area of 1.89 million hectares, including 75,000 ha of certified organic cultivation [8]. Today, the olive sector employs 269,000 farmers, or 57% of all farmers, and contributes to 45% of agricultural exports, averaging 120,000 tons per year [9, 10].

Research on Tunisian olive germplasm began in the twentieth century. Initially, studies focused on the morphological and biochemical traits of a limited number of olive varieties, such as the most widely cultivated "Chetoui" and "Chemlali" and some minor cultivars, such as "Oueslati", "Besbessi", and "Gerboui" [11–15]. Over time, studies moved to using molecular markers, which allowed the identification of more than 83 varieties distributed throughout Tunisia [16, 17] and the registration of 56 cultivars in the national catalogue [18]. However, given that Tunisia harbours a great genetic diversity of *Olea europaea* L., this number falls far short of reality. Many monumental trees whose age exceeds thousand years have never been studied for their oil composition and quality. Mnasri et al. [12, 19] studied their geographical distribution across the country and their thriving in desert and arid areas and explained their survival by people's belief in their sacredness. Using morphological and molecular data, the authors pointed out a great genetic diversity that could be used in olive breeding programs. Thus, there is an urgent need to protect and study these thousand-year-old olive trees to prevent their extinction in the face of constantly intensified agriculture. For decades, Tunisian olive oil was sold in large quantities to other countries to be blended with other oils under European brand names. Today, Tunisia is making considerable efforts to produce and sell oils under Tunisian brands that are competitive on the world market, such as Teboursouk olive oil, which is registered as a protected designation of origin (PDO) in the international register of designations of origin [20].

The study aimed to examine the quality of 28 oils from millennial olive trees from different regions of Tunisia to obtain information on their oil quality and composition. The results will help us understand the flavor and quality of the oils consumed by our ancestors and select those whose quality complies with international standards by a composition rich in oleic acid, carotenes, chlorophyll, and polyphenols, to integrate them into the national breeding programs to increase the nutritional value of Tunisian oil and the competitiveness of the Tunisian olive industry on an international scale.

Materials and methods

Olive samples

The National GeneBank of Tunisia carried out extensive surveys throughout Tunisia and found 28,000-year-old olive trees of impressive size and appearance in the mountainous and arid inland areas known for their difficult climatic conditions (Figs. 1, 2). Trees at each sampling site were selected on the basis of the growth habit, structure, trunk thickness, using information from local growers and trunk dimensions as indicators of tree age according to [21], selecting trees with a girth of 6–8 m measured at 1.3 or 1.4 m above the ground (Table 1).

Genotyping of olives

Fresh, healthy leaves were collected from each plant in July 2021, genomic DNA was extracted according to [22], and DNA quality and concentration were checked on 0.8% agarose gel and a Nano Drop TM ND2000c (Thermo Scientific, MA, USA) spectrophotometer. DNA concentrations were normalized to 50 ng/µL using 0.1 X TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA) and polymerase chain reactions were carried out using a set of 10 highly informative SSR markers consolidated for olive genotyping [23]. PCR reactions were performed in a final volume of 12.5 µL and contained 1X Dream Taq buffer, 0.15 mM dNTP, 0.25 µM primer mix, 0.3 U Dream Taq, and 50 ng genomic DNA. Amplicons were separated using the automatic capillary sequencer ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with the internal size standard GeneScan 600 LIZ (Applied Biosystems). Allele size was estimated using GeneMapper v.5.0 software (Applied Biosystems, Foster City, CA, USA).

For three consecutive harvest years (2018–2021), 1 kg of fresh olives was hand-picked from different parts of each tree at full and equal ripeness index (RI = 3), based on the assessment of olive skin and pulp color [24]. All fruits were visually inspected and damaged fruits or those affected by pests and diseases were discarded. The olives were washed and processed within 24 h of harvest in a laboratory mill of the National GeneBank, Tunisia (MC2 Ingenieria Y Sistemas, Seville, Spain) equipped with a metal crusher, a mixer, and a basket centrifuge to obtain extra virgin olive oils (EVOO) through a cold extraction process. The mill was washed between each batch of olive fruits. The oil samples obtained were stored in dark glass bottles at 4 °C until they were used for the analyses, which were carried out in triplicate.

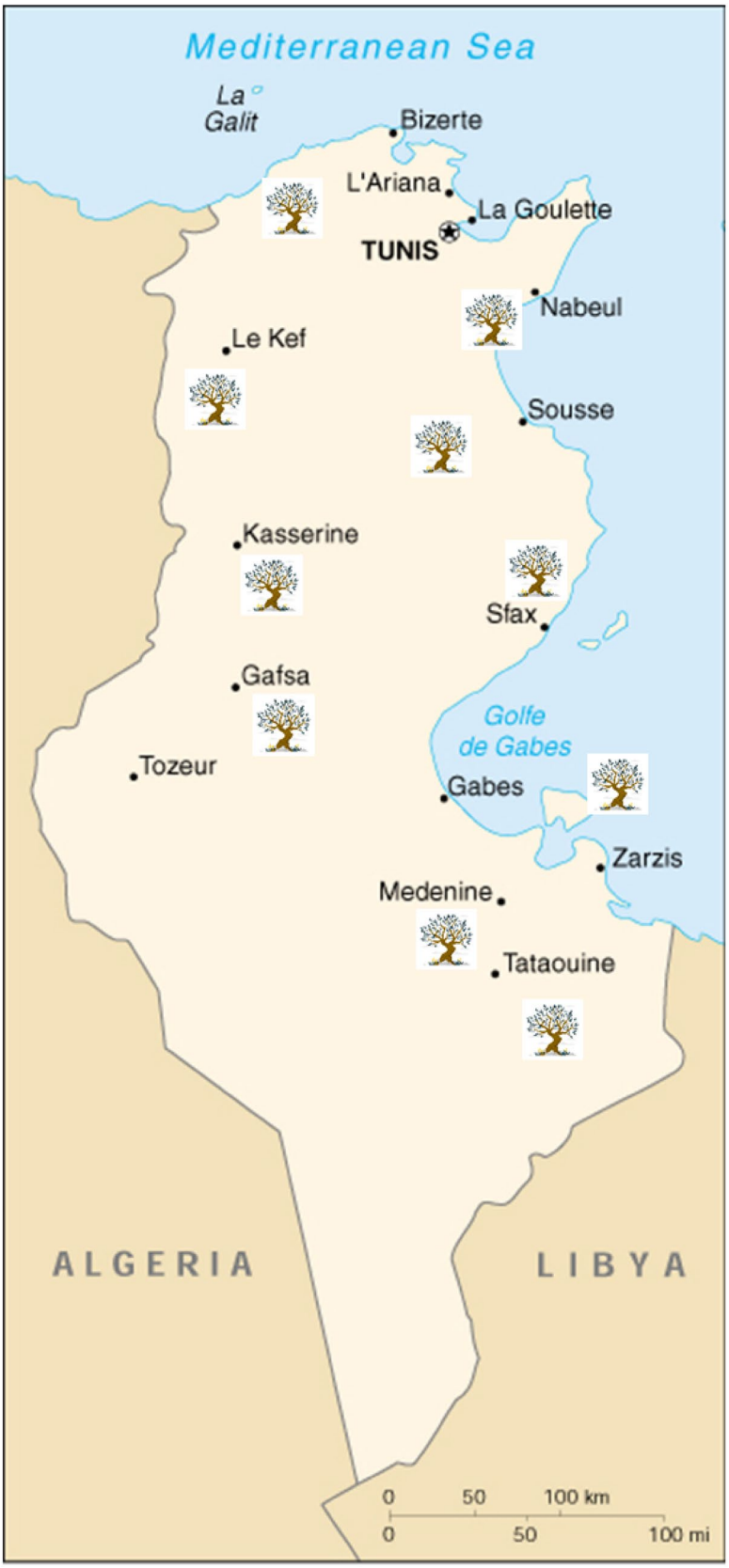

Fig. 1 Geographical distribution of the analyzed millennial olive trees

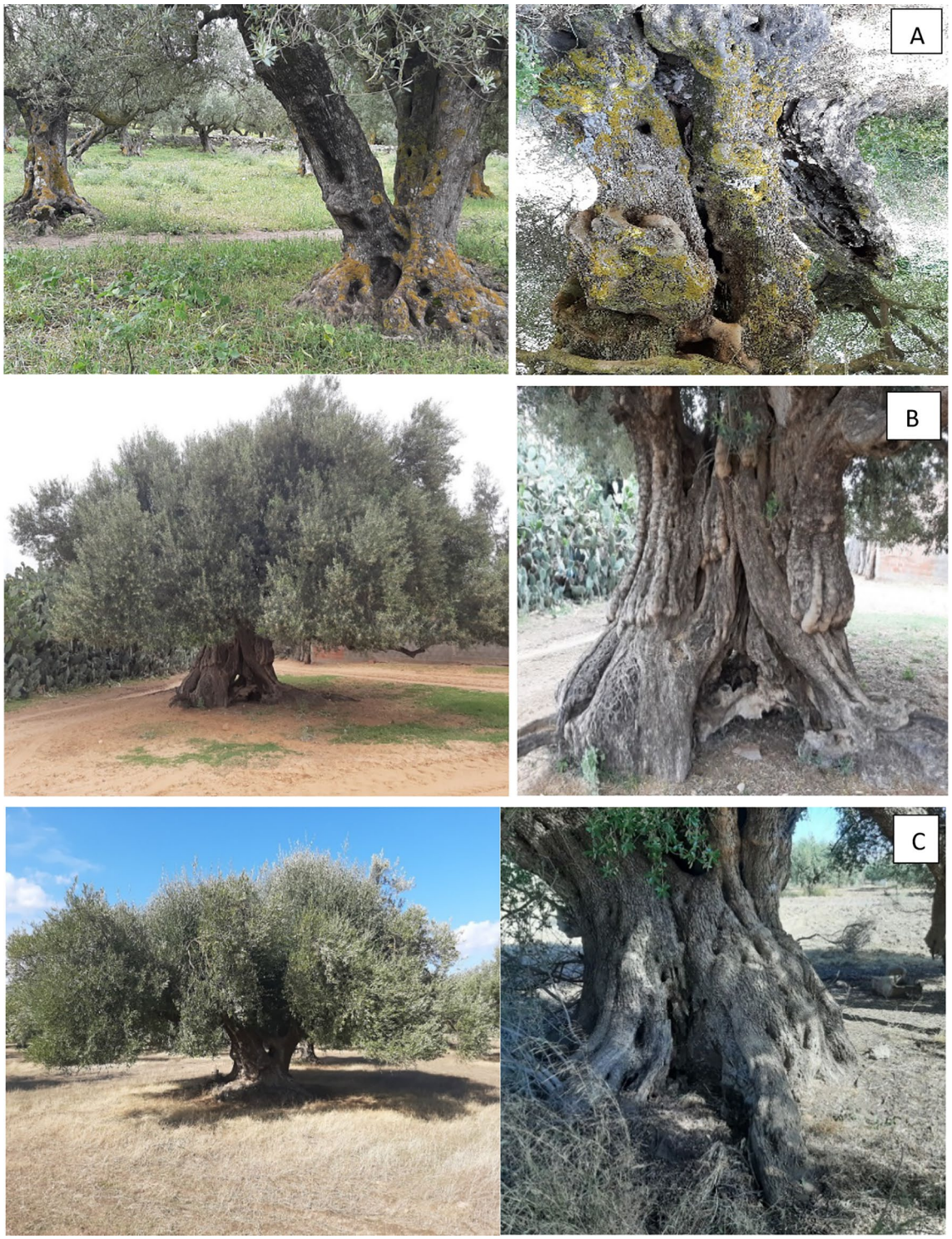

Fig. 2 Some of the monumental olive trees of Tunisia located in **A** Beja (North), **B** Kairouan (Center), and **C** Gafsa (South)

Physiochemical quality indices of the oil

Free fatty acids and K_{232} and K_{270} extinction coefficients spectrophotometric were determined according to the official methods described in the European Union standard methods Regulations 2568/91 and the subsequent amendments by the Commission of the European Union.20 [25]. All parameters were determined in triplicate for each sample.

Table 1 Origin and use of the millennium olive cultivars under investigation

Sample	Region of collection site	Locality	Use
M1	Haouria	Nabeul (North)	Table
M2	Mednine	Mednine (Center)	Oil
M3	Sfax	Sfax (Center)	Oil
M4	Zahret Medyen	Béja (North)	Oil and table
M5	Gafsa	Gafsa ((South)	Oil and table
M6	Gafsa	Gafsa (South)	Oil and table
M7	Zarzis	Mednine (South)	Oil and table
M8	Mountain of Kesra	Siliana (Center)	Oil
M9	Mountain of Kesra	Siliana ((Center)	Oil and table
M10	Mountain of Kesra	Siliana (Center)	Oil
M11	Mountain of Kesra	Siliana (Center)	Oil
M12	Mountain of Kesra	Siliana (Center)	Oil and table
M13	Testour	Béja ((North)	Oil and table
M14	Testour	Béja (North))	Oil and table
M15	Testour	Béja (North)	Oil and table
M16	Haouria	Nabeul (North)	Oil
M17	Testour	Béja (North)	Oil and table
M18	Sbeitla	Kasserine (Center)	Oil
M19	Slimen	Nabeul (North)	Oil
M20	Sbeitla	Kasserine (Center)	Oil and table
M21	El Alaa	Kairouan (Center)	Oil
M22	El Alaa	Kairouan (Center))	Oil
M23	Ben Gardène	Tataouine (South)	Oil
M24	Ben Gardène	Mednine (South)	Oil
M25	Tataouine	Tataouine (South)	Oil
M26	Jerba	Mednine (South)	Oil
M27	Sbeitla	Kasserine (Center)	Oil and table
M28	Zahret Medyen	Béja (North)	Table

Chlorophyll and carotenoid pigments

The content of chlorophylls and carotenoids pigments was expressed as mg of pheophytin *a* or lutein per kg of oil. Carotenoids are conjugated terpene compounds in various forms (α, β and γ) of which the β-carotene, the biochemical precursor of vitamin A, is the most abundant. Pigments were determined according to the method described in [26] by measuring the absorbance at 670 and 470 nm of a mixture obtained by dissolving 7.5 g of oil in cyclohexane to a final volume of 25 ml. The absorbance was determined as $(A_{670}*10^6)/(613*100*d)$ for chlorophyll and as $(A_{470}*10^6)/(2000*100*d)$ for carotenoid, where '*A*' is the absorbance and '*d*' is the thickness of the spectrophotometer cell (1 cm).

Phenolic profile

Phenolic compounds' extraction and purification

The phenolic fraction was extracted according to [27] by mixing 2.5 g of fresh oil with 250 ml of the internal standard solution (15 ppm syringic acid in methanol). The emulsion was vortexed for 10 min and evaporated in a rotary evaporator at 35 °C under vacuum. The aliquot was dissolved in 6 ml of hexane. The phenols were purified according to [28]. Diol SPE cartridges (Supelco Co., Bellefonte, PA, USA) were placed in a vacuum elution apparatus to refine the phenolic phase. The cartridge was conditioned by the sequential addition of 6 ml methanol and 6 ml hexane. The oil solution was injected into the column after releasing the vacuum. The solvent was drawn through the column leaving the sample and standard on the solid phase. The sample container was washed with 6 ml hexane followed by 4 ml hexane/ethyl acetate (85:15, v/v), and the solvents were removed from the cartridge and discarded. The phenolic phase was eluted from the column with 15 ml methanol, vortexes for 5 min, and the solvent removed on a low-speed rotary evaporator under vacuum at room temperature. Finally, the residue was dissolved in 250 ml of methanol/water (1:1, vol/vol).

Phenolic composition

The total phenolic content, expressed as mg gallic acid equivalent (GAE) per kg EVOO, was determined by the spectrophotometric Folin–Ciocalteu method of Singleton and Rossi [29]. Gallic acid was used in the external calibration at different concentrations, including 0.00, 0.25, 0.50, 0.75, and 1 mM. After 30 min, the solution was tested to determine its absorbance at 750 nm using a UV–Vis spectrophotometer. The total phenolic content was calculated as mM gallic acid equivalent (mM GAE) using the calibration curve. The phenolic compounds were identified using the Agilent 1100 liquid chromatography system equipped with an automatic injector, a column oven (Waters Co., Milford, MA, USA), and a diode array UV detector. A Spherisorb S3 ODS2 column (250*4.6 mm i.e., 5 µm particle size) was maintained at 30 °C, with a flow rate of 1.0 ml/min and an injection volume of 20 µl. The mobile phases consisted of solvent A (water/acetic acid 95:5, vol/vol), methanol (B), and acetonitrile (C). The following multi-step linear gradient was applied over a period of 50 min: it started at 95% A, 2.5% B, and 2.5% C and changed to 34% A, 33% B and 33% C. The phenols were quantified at 280 nm using syringic acid as an internal standard, and the response factors were determined by comparing their retention times with maximum absorbance according to [29]. Simple phenols were identified

using pure standards injected under the same conditions as the olive oil extracts. Complex phenols were tentatively identified with two replicate analyses based on their retention times, and phenolic compound concentrations expressed as mg/kg syringic acid.

Fatty acid composition

The fatty acid composition was determined by gas chromatography according to the International Olive Council [30]. Fatty acid methyl esters (FAME) were dissolved by mixing 0.2 g of oil in 3 ml of hexane. The solution was mixed with 0.4 ml of 2N methanolic potassium hydroxide before being analyzed by gas phase chromatography. The carrier gas was nitrogen (1 ml/min), the injector and detector (FID) temperatures were 240 °C and 260 °C, respectively, and the oven temperature was 210 °C. The spillage ratio was 1/5 and the injection volume was 1 µl. Fatty acids were identified by comparing each sample with fatty acid methyl ester standards.

Statistical analysis

Statistical analysis was performed using XLSTAT v. 6.1 software (Addinsoft, Paris, France) and the statistical package SPSS software (IBM version 17). Results were reported as tables of mean values and standard deviations. One-way analysis of variance (ANOVA) was used to evaluate the effects of the genotype and the growing location on the composition of the oil and its quality parameters. The significance of differences at 1% ($p < 0.01$) between mean values was determined using Turkey's test.

Results

Quality parameters

The results for quality parameters of the analyzed EVOO oils are presented in Table 2. At the fruit ripening stage RI = 3, all the oils except M24 and M27 had a free acidity content lower than the upper limit of 0.8% which is the threshold limit established for the "Extra Virgin Olive Oil" category. The specific extensions' values of K_{232} and K_{270} were ≤ 2.5 and ≤ 0.22, respectively, complying with the limits prescribed in [25].

Chlorophyll and carotenoids

The chlorophyll content of the analyzed EVOOs ranged from 0.28 (sample M1) to 22.1 ppm (sample M27) with significant differences among the oils (p value < 0.01) (Table 2). Most of oils had low-to-medium content (average 3.16 ppm), in line with other Tunisian oils [31–33], but oils M6, M19, M20, and M27 had a very high chlorophyll content (17.2 to 22.1 ppm (Table 2). Carotenoids also varied significantly (0.21–6.64 ppm), and were > 1 ppm, which is the threshold requested for an effective inhibition of photo-oxidation, in most of oils. Although many factors influence the pigment profile of olive fruits, such as ripening degree and growing conditions [34–36], four oils, M9, M19, M20, and M27, had a carotene content > 3 ppm.

Polyphenols

In the studied oils, the polyphenol content, expressed as mg gallic acid equivalent (GAE) per kg EVOO, varied significantly ($p < 0.01$) ranging from 91.2 to 1632 mg/kg (Table 3). Several oils had content > 500 mg/kg, in particular those originating from the arid regions of Kesra (M10, M11, M12), El Alaa (M21, M22), and Gafsa and Medenine (M3, M5, M7, M24, and M25) (Table 3).

As for the phenolic composition, chromatography revealed 14 compounds (Table 3). Among the monomeric phenols, tyrosol (p-hydroxyphenyl ethanol: p-HPEA), hydroxytyrosol (3,4-dihydroxy phenyl ethanol: 3,4-DHEA), and their derivatives were the most abundant. These phenols have important biological activities as free radical scavengers and metal-chelators [37, 38] and show a variety of beneficial effects on human health, including cardio protective, anticancer, neuroprotective, antimicrobial, and endocrine effects [39]. Tyrosols reached 55.7 mg/kg in sample M1, and hydroxytyrosol reached 34.36 mg/kg in M12.

Among the complex phenols, the aldehyde form of elenolic acid combined with hydroxytyrosol (oleocanthal, 3,4-DHPEA-EDA) or tyrosol (oleuropein, p-HPEA-EDA), the ligstroside aglycone (p-HPEA-EA), and an isomer of the oleuropein aglycone (3,4-DHPEA-EA) are the most abundant secoiridoid fractions in virgin olive oil [40–44]. The analyzed oils differed significantly in the content of oleocanthal and oleuropein, reaching, in sample "M11", the content of 337.33 mg/kg and 225.33 mg/kg, respectively. This oil was also the richest in oleuropein aglycone (668 mg/kg) and ligustroside aglycone (150.66 mg/kg), two components very important in the metabolism of the human body by reducing the levels of reactive oxygen species [45, 46]. Other phenols, such as vanillic acid, vanillin, p-coumaric acid, and ferulic acid, were also found, although in low amounts (0.001–5.35 mg/kg). Finally, the lignans pinoresinol and acetoxypinerosol varied from 0.77 to 17.85 mg/kg and from 0.001 to 6.81 mg/kg, respectively, but they were > 12 mg/kg in samples M2, M3, M4, M11, and M28. These compounds belong to the phytoestrogen family and have beneficial health effects [47]. In clinical studies, it has been observed

Table 2 Mean values and standard deviations (SD) of quality parameters of olive oils obtained from fruits at Maturity index = 3. Free acidity, UV spectrophotometer indices, and total chlorophylls and carotenes' pigments' content are listed. In the same column, the means marked by different lowercase letters differ significantly (Tukey's test, p 0.05).

Sample	Free acidity (g/100 g)	K_{232}	K_{270}	Chlorophylls	Carotenes
M1	0.3 ± 0.014jk	2.44 ± 0.0.3hi	0.15 ± 0.01gh	0.28 ± 0.024a	0.56 ± 0.055bc
M2	0.41 ± 0.008lm	1.75 ± 0.017cd	0.11 ± 0.03cd	7.41 ± 0.002m	0.56 ± 0.05bc
M3	0.33 ± 0.04kl	2.12 ± 0.07hi	0.14 ± 0.001fg	0.29 ± 0.02a	0.47 ± 0.04b
M4	0.34 ± 0.012kl	2.28 ± 0.03hi	0.21 ± 0.003jk	5.62 ± 0.03k	0.21 ± 0.018a
M5	0.27 ± 0.009jk	2.32 ± 0.07hi	0.15 ± 0.004gh	2.61 ± 0.06fg	0.42 ± 0.064b
M6	0.32 ± 0.021kl	2.47 ± 0.01hi	0.21 ± 0.004k	17.2 ± 0.2o	1.6 ± 0.067hi
M7	0.36 ± 0.04kl	2.25 ± 0.08hi	0.16 ± 0.007gh	8.51 ± 0.05n	0.79 ± 0.016d
M8	0.19 ± 0.02de	1.41 ± 0.0a	0.13 ± 0.01ef	4.23 ± 0.16j	2.19 ± 0.08kl
M9	0.21 ± 0.02fg	1.49 ± 0.01a	0.13 ± 0.02ef	6.22 ± 0.16l	3.82 ± 0.07o
M10	0.24 ± 0.01gh	1.77 ± 0.06de	0.13 ± 0.01ef	1.15 ± 0.06b	1.07 ± 0.04ef
M11	0.18 ± 0.01cd	1.89 ± 0.04fg	0.21 ± 0.01k	2.24 ± 0.15de	1.72 ± 0.07hi
M12	0.26 ± 0.01ij	1.89 ± 0.04fg	0.21 ± 0.01jk	3.77 ± 0.12i	2.67 ± 0.06n
M13	0.14 ± 0.04bc	2.08 ± 0.07gh	0.10 ± 0.01ab	3.13 ± 0.05h	1.40 ± 0.01g
M14	0.19 ± 0.04de	1.7 ± 0.05de	0.12 ± 0.02de	3.83 ± 0.15i	2.33 ± 0.05ml
M15	0.1 ± 0.02a	1.47 ± 0.29a	0.10 ± 0.01ab	2.5 ± 0.2ef	1.8 ± 0.1ij
M16	0.2 ± 0.01ef	1.41 ± 0.07a	0.11 ± 0.01bc	2.9 ± 0.1gh	2,0 ± 0.1jk
M17	0.25 ± 0.02hi	1.82 ± 0.02ef	0.12 ± 0.01de	2.06 ± 0.11cd	1.08 ± 0.07f
M18	0.35 ± 0.01kl	1.6 ± 0.05ab	0.09 ± 0.002a	1.33 ± 0.02b	0.40 ± 0.02ab
M19	0.13 ± 0.001ab	2.22 ± 0.7hi	0.19 ± 0.1ij	19.75 ± 0.06p	5.60 ± 0.2p
M20	0.77 ± 0.025o	1.66 ± 0.015bc	0.10 ± 0.0017ba	22.1 ± 0.1q	6.64 ± 0.03q
M21	0.29 ± 0.01jk	1.85 ± 0.05fg	0.13 ± 0.003fg	2.86 ± 0.05gh	0.86 ± 0.02de
M22	0.63 ± 0.05n	1.87 ± 0.02fg	0.12 ± 0.001cd	3.93 ± 0.11ij	0.72 ± 0.02cd
M23	0.46 ± 0.01m	1.76 ± 0.015de	0.18 ± 0.011hi	1.05 ± 0.005b	2.54 ± 0.01mn
M24	1.58 ± 0.11q	2.31 ± 0.15hi	0.19 ± 0.01jk	2.05 ± 0.005cd	2.33 ± 0.005ml
M25	0.28 ± 0.005jk	2.27 ± 0.19hi	0.10 ± 0.001ba	2.05 ± 0.005cd	2.33 ± 0.005ml
M26	0.25 ± 0.01hi	2.6 ± 0.005hi	0.18 ± 0.005hi	1.76 ± 0.05c	1.51 ± 0.005gh
M27	1.30 ± 0.1p	1.83 ± 0.02fg	0.12 ± 0.001cd	22.1 ± 0.1q	6.62 ± 0.02q
M28	0.61 ± 0.01n	2.09 ± 0.01gh	0.19 ± 0.004jk	4.16 ± 0.05j	2.23 ± 0.05l

that women with a high intake of plant lignans have a lower risk of postmenopausal breast cancer, especially the ER + / PR + subtype, as well as a reduction in the expression of the tyrosine kinase proteins FASN and HER2, which are directly involved in cancer processes [48].

Fatty acid profile

The fatty acid composition (%) of olive oils is summarized in Table 4. The oils studied showed remarkable differences in the content and composition of the major fatty acids ($p < 0.001$). Oleic acid was the most abundant (average 67.36%), followed by palmitic acid (14, 6%), linoleic acid (C18:2), and palmitoleic acid (average 1, 39%). Among the saturated fatty acids (SFA), palmitic acid was the most abundant, followed by stearic acid (2.55%) and arachidic acid (0.31%).

Polyunsaturated fatty acids (PUFAs) were mainly represented by linoleic acid, which ranged from 5.05% (M5) to 22.74% (M13), and linolenic acid from 0.1% (in M1) to 0.91% (in M14). Nine oils (M5, M6, M8, M9, M10, M11, M14, M15, and M21) had an oleic acid/linoleic acid ratio > 7, which is the threshold which correlates with a high quality for olive oil [49, 50].

Discussion

Since the twentieth century, efforts to develop the quality of olive oil in Mediterranean countries have greatly increased, extending the study to thousands of trees, along the Aegean Sea. In Crete, the largest Greek island exists a 3000-year-old olive tree in Kavousi. It is an olive tree of exceptional size with a 14.2 m perimeter and nearly 5 m in diameter [51]. Italian CNR researchers on the olive genetic resources proved the existence of Millennium olive trees in the Mount of Palestine and Urla, a small Turkish peninsula [52]. The Spanish researchers consider that the ancient olives from southern Spain constitute a priceless reservoir of genetic

Table 3 Phenolic compound composition of the studied olive oils

Cultivar	Hydroxy-tyrosol	Tyrosol	Vanillic acid	Vanillin	*P*-Coumaric acid	3.4 DHPEA-AC	Ferulic acid
M1	2.71 ± 0.01ef	55.7 ± 5.75	2.48 ± 0.18l	1.28 ± 0.03g	1.18 ± 0.02de	1.43 ± 0.00i	1.46 ± 0.01ef
M2	14.8 ± 0.5l	12.33 ± 0.35gh	0.28 ± 0.04cd	0.29 ± 0.01cd	0.4 ± 0.03ab	0.64 ± 0.03f	0.34 ± 0.05bc
M3	6.8 ± 0.2i	5.2 ± 0.10de	1.5 ± 0.01j	4.8 ± 0.1j	2.81 ± 0.01f	6.05 ± 0.05m	7.15 ± 0.15j
M4	14.5 ± 0.2l	10.5 ± 0.2gh	0.51 ± 0.01f	0.38 ± 0.02d	0.65 ± 0.05bc	1.38 ± 0.03hi	0.65 ± 0.05cd
M5	29 ± 0.00o	14 ± 0.2hi	0.35 ± 0.05b	0.6 ± 0.05[e]	0.35 ± 0.05ab	0.35 ± 0.05e	32 ± 0.00k
M6	8.8 ± 0.1k	12 ± 0.1gh	0.65 ± 0.05h	2.3 ± 0.1h	0.75 ± 0.05cd	3.7 ± 0.01k	31.73 ± 0.64k
M7	26.5 ± 0.5n	21.5 ± 0.5j	0.89 ± 0.01i	0.7 ± 0.05e	2.05 ± 0.05ef	0.9 ± 0.05g	3.2 ± 0.1h
M8	3.31 ± 0.01fg	4.99 ± 0.01de	0.26 ± 0.00bc	0.15 ± 0.00bc	0.14 ± 0.00a	0.18 ± 0.00cd	1.43 ± 0.01ef
M9	4.66 ± 0.05h	5.12 ± 0.06de	0.35 ± 0.01de	0.18 ± 0.00bc	0.19 ± 0.00a	0.19 ± 0.01d	1.78 ± 0.01fg
M10	8.36 ± 0.05k	5.78 ± 0.02ef	0.25 ± 0.00bc	0.2 ± 0.00bc	0.24 ± 0.00ab	0.31 ± 0.00e	5.35 ± 0.05i
M11	18.66 ± 0.58m	8.5 ± 0.01fg	0.14 ± 0.005ab	0.11 ± 0.01ab	0.46 ± 0.01ab	0.38 ± 0.00e	1.18 ± 0.01de
M12	34.36 ± 0.1p	27.7 ± 0.3k	0.51 ± 0.11fg	0.19 ± 0.00bc	0.38 ± 0.02ab	0.38 ± 0.00e	2.13 ± 0.2g
M13	0.21 ± 0.01a	0.95 ± 0.01ab	0.42 ± 0.00ef	0.05 ± 0.00ab	0.86 ± 0.1cd	0.05 ± 0.05a	0.17 ± 0.00ab
M14	0.06 ± 0.00a	0.66 ± 0.01a	0.33 ± 0.00de	0.22 ± 0.00cd	0.34 ± 0.00ab	0.08 ± 0.00bc	0.09 ± 0.00a
M15	1.13 ± 0.00c	5.43 ± 0.04de	0.45 ± 0.00ef	0.06 ± 0.00ab	0.71 ± 0.00bc	0.06 ± 0.00ab	0.14 ± 0.00ab
M16	1.05 ± 0.01bc	2.07 ± 0.01cd	0.42 ± 0.00ef	0.06 ± 0.00ab	0.3 ± 0.00ab	0.3 ± 0.00e	0.3 ± 0.00bc
M17	0.05 ± 0.00a	0.68 ± 0.00a	0.7 ± 0.00h	0.17 ± 0.00bc	0.4 ± 0.00ab	0.4 ± 0.00e	0.47 ± 0.00bc
M18	0.03 ± 0.00a	0.76 ± 0.01a	0.4 ± 0.00ef	0.19 ± 0.00bc	0.32 ± 0.00ab	0.07 ± 0.00ab	0.3 ± 0.00bc
M19	0.5 ± 0.00ab	1.38 ± 0.01b	0.13 ± 0.01ab	0.07 ± 0.04ab	0.07 ± 0.00a	0.04 ± 0.00a	0.09 ± 0.00a
M20	0.34 ± 0.00a	4.26 ± 0.18cd	0.91 ± 0.00i	0.06 ± 0.00ab	0.56 ± 0.00bc	0.07 ± 0.00ab	0.32 ± 0.00bc
M21	0.37 ± 0.00a	0.34 ± 0.00a	0.07 ± 0.00a	1.04 ± 0.00f	0.22 ± 0.00ab	0.001 ± 0.00a	0.1 ± 0.00bc
M22	0.61 ± 0.00ab	0.6 ± 0.00cd	0.22 ± 0.00bc	0.28 ± 0.00cd	0.32 ± 0.00ab	0.09 ± 0.00cd	0.79 ± 0.00cd
M23	0.15 ± 0.00a	0.27 ± 0.00a	0.03 ± 0.00a	0.048 ± 0.00a	0.8 ± 0.004cd	0.05 ± 0.00ab	0.17 ± 0.00ab
M24	1.56 ± 0.01cd	3.06 ± 0.08cd	0.64 ± 0.00gh	0.056 ± 0.01ab	1.03 ± 0.00de	0.15 ± 0.00cd	0.32 ± 0.00bc
M25	2.4 ± 0.06[e]	3.86 ± 0.08cd	1.47 ± 0.01j	4.86 ± 0.04j	2.3 ± 0.06ef	5.9 ± 0.06l	1.17 ± 0.01de
M26	7.6 ± 0.06j	16.56 ± 0.15i	0.78 ± 0.02hi	0.62 ± 0.00e	0.98 ± 0.05de	0.9 ± 0.01g	0.81 ± 0.04cd
M27	3.5 ± 0.00g	9.63 ± 0.11fg	2.56 ± 0.04l	3.5 ± 0.13i	1.78 ± 0.01ef	1.29 ± 0.02h	3.4 ± 0.06h
M28	2.16 ± 0.04de	3.96 ± 0.00cd	1.84 ± 0.04k	0.60 ± 0.06e	2.49 ± 0.01ef	3.51 ± 0.05j	1.82 ± 0.1fg
Average	1.91	4.39	0.86	1.11	1.05	1.2	0.89

Cultivar	3.4-DHPEA-EDA	p-HPEA-AC	p-HPEA-EDA	Pinoresinol	Acetoxy-pinerosol	Oleuropein aglycon	Ligustroside aglycon	Total phenol (mg/kg)
M1	0.87 ± 0.11a	0.64 ± 0.06hi	20 ± 0.5h	5.31 ± 0.38ef	0.64 ± 0.06d	11.94 ± 0.11g	23.9 ± 0.1g	337 ± 1.33g
M2	1.21 ± 0.01a	0.33 ± 0.04ef	1.18 ± 0.02a	12.5 ± 0.5j	0.33 ± 0.04c	125 ± 1n	60 ± 0.4j	410 ± 0.66h
M3	2.2 ± 0.1a	8.53 ± 0.05l	5.3 ± 0.6cd	13 ± 0.2k	8.53 ± 0.05m	208 ± 2p	139 ± 1l	628 ± 3.33l
M4	4.1 ± 0.1a	0.3 ± 0.05ef	4 ± 0.5bc	3.3 ± 0.1c	0.3 ± 0.05c	89 ± 0.5l	26 ± 1h	460 ± 1.33i
M5	2.63 ± 0.35a	0.286 ± 0.03ef	3.6 ± 0.2bc	1.06 ± 0.01a	0.28 ± 0.03c	76 ± 0.5k	21.6 ± 0.2f	527 ± 10.66j
M6	33 ± 0.5a	1.22 ± 0.02j	132 ± 1o	1.77 ± 0.07a	1.22 ± 0.02h	4.1 ± 0.1a	20.5 ± 0.5f	430.66 ± 3.77hi
M7	12 ± 0.2a	0.38 ± 0.00fg	190 ± 2p	2.7 ± 0.1b	0.38 ± 0.00c	6.5 ± 0.3f	27 ± 0.5h	670 ± 10m
M8	185 ± 1b	0.34 ± 0.01fg	28.9 ± 0.11k	6.67 ± 0.02g	1.09 ± 0.01g	17.9 ± 0.17h	4.1 ± 0.1cd	256 ± 1.33f
M9	232 ± 40.7b	0.4 ± 0.02fg	39.23 ± 0.66l	8.97 ± 0.02i	1.42 ± 0.02i	30.6 ± 1.21j	5.66 ± 0.05e	354 ± 2.66g
M10	285 ± 24.26c	0.85 ± 0.011i	127.33 ± 0.57n	3.35 ± 0.05b	2.06 ± 0.015j	105.33 ± 0.57m	27.26 ± 0.25h	586 ± 6kl
M11	337.33 ± 2.51c	4.56 ± 0.05k	225.33 ± 0.57k	15.1 ± 0.1l	6.16 ± 0.15l	668 ± 1l	150.66 ± 1.15m	1632 ± 4.66n
M12	142.33 ± 2.08b	0.48 ± 0.01gh	121 ± 1m	5.06 ± 0.11e	4.17 ± 0.02k	210.33 ± 0.57q	75.66 ± 0.57k	621 ± 2.66kl
M13	0.39 ± 0.5a	0.05 ± 0.05ab	4.56 ± 0.15cd	4.6 ± 0.1d	0.08 ± 0.00ab	2.28 ± 0.25cd	0.90 ± 0.011a	185.66 ± 10.44cd
M14	0.13 ± 0.00a	0.05 ± 0.00ab	2.71 ± 0.01ab	3.14 ± 0.00c	0.03 ± 0.00ab	0.89 ± 0.01ab	0.37 ± 0.01a	254 ± 2f
M15	0.9 ± 0.00a	0.09 ± 0.00de	2.56 ± 0.00ab	5.1 ± 0.33e	0.03 ± 0.00ab	2.69 ± 0.01de	0.69 ± 0.00a	261 ± 2.66f
M16	0.55 ± 0.00a	0.15 ± 0.00de	5.26 ± 0.04cd	3.9 ± 0.26cd	0.11 ± 0.00ab	2.33 ± 0.02cd	0.37 ± 0.00a	210 ± 3.33a
M17	1.16 ± 0.00a	0.1 ± 0.00de	2.5 ± 0.00ab	7.1 ± 0.00h	0.13 ± 0.00b	3.02 ± 0.00de	0.38 ± 0.00a	150 ± 0.00bc
M18	1.8 ± 0.0a	0.36 ± 0.00fg	7.32 ± 0.17e	3.46 ± 0.00c	0.05 ± 0.00ab	1.48 ± 0.02bc	0.19 ± 0.00a	125.54 ± 0.6ab
M19	12.04 ± 0.18a	0.05 ± 0.00ab	26.4 ± 0.1i	2.61 ± 0.06b	0.001 ± 0.00a	7.32 ± 0.05f	2.54 ± 0.00b	504.91 ± 3.1j
M20	0.17 ± 0.00a	0.07 ± 0.00cd	3.65 ± 0.02bc	5.09 ± 0.00e	0.001 ± 0.00a	0.96 ± 0.00ab	0.42 ± 0.00a	91.2 ± 0.73a

Table 3 (continued)

Cultivar	3.4-DHPEA-EDA	p-HPEA-AC	p-HPEA-EDA	Pinoresinol	Acetoxy-pinerosol	Oleuropein aglycon	Ligustroside aglycon	Total phenol (mg/kg)
M21	39.63 ± 0.48a	0.01 ± 0.00a	27.03 ± 0.00i	1.28 ± 0.04a	0.001 ± 0.00a	3.5 ± 0.06de	0.67 ± 0.01a	668.95 ± 10.18m
M22	15.63 ± 0.11a	0.06 ± 0.00bc	6.25 ± 0.1de	4.44 ± 0.01d	0.01 ± 0.00a	4.42 ± 0.00e	0.001 ± 0.00a	242.94 ± 1.16ef
M23	0.9 ± 0.01a	0.04 ± 0.00ab	4.03 ± 0.00bc	1.260.01a	1.26 ± 0.01ab	0.35 ± 0.00a	26.83 ± 0.17h	145.1 ± 1.34b
M24	6.48 ± 0.02a	0.23 ± 0.00ef	5.16 ± 0.04cd	3.13 ± 0.11c	0.36 ± 0.00c	6.61 ± 0.06f	27.11 ± 0.06h	681.11 ± 0.01m
M25	2.71 ± 0.07a	0.85 ± 0.02i	10.21 ± 0.05f	5.82 ± 0.09f	0.79 ± 0.01ef	165.96 ± 0.06o	40.1 ± 0.06i	586 ± 25.33k
M26	4.02 ± 0.11a	0.76 ± 0.04hi	28.73 ± 0.17jk	2.86 ± 0.04bc	0.7 ± 0.00a	28.5 ± 0.33i	5.1 ± 0.06a	317.39 ± 4.13g
M27	25.76 ± 0.04a	0.91 ± 0.06i	11.43 ± 0.05f	5.05 ± 0.1e	0.87 ± 0.00f	30.16 ± 0.55ij	3.2 ± 0.13bc	323.66 ± 5.77g
M28	11.98 ± 0.01a	0.92 ± 0.00ij	14.96 ± 0.17g	17.85 ± 0.03m	0.9 ± 0.00f	7.19 ± 0.19f	2.51 ± 0.00b	449.7 ± 25.2i
Average	11.92	0.39	13.78	4.93	0.48	25.49	10.84	401.09

diversity [53]. In Tunisia, olive trees were first introduced by Phoenicians following the Second Punic War [54].

This study presents the primary analysis of millennium olives in Tunisia. Our preliminary analyses have shown that oils from Tunisian millennial olive trees are of exceptional quality and very rich in polyphenols, carotenes, and monounsaturated fatty acids. The free acidity was below than the upper limit of 0.8% set for the "Extra Virgin Olive Oil" category, and the K_{232} and K_{270} extensions were ≤ 2.5 and ≤ 0.22, respectively. The small variations observed in these parameters confirm the low influence of location and genotype on the primary and secondary oxidation level [55]. In fact, fruit ripeness, careless harvesting, and storage conditions strongly influence oil quality, as triglycerides are gradually hydrolyzed in ripe fruits and poor storage conditions expose the oils to enzymatic activity that increases free acidity and eventually gives the oil an unpleasant taste [56].

Chlorophylls and carotenoids are the main color pigments in olive oil, responsible for the color, green for chlorophylls and yellowish for carotenoids, and taste of the oils [32]. They are directly related to the quality of this food and play an essential role in the oxidative activity of the oil acting as antioxidants in the dark and as prooxidants when exposed to light [24, 57]. Most of the oils had low-to-medium chlorophylls content (average 3.16 ppm), which is consistent with other Tunisian oils [32–34], but M6, M19, M20, and M27 had a very high levels of chlorophyll (17.2 to 22.1 ppm) and carotenoids (> 3), which makes them interesting for direct use to improve the color and "fruity" note of olive oil in new blends [58], and makes an important contribution to consumers acceptance [24].

Polyphenols are a very important component of the potential benefits of olive oil, as they have a strong antioxidant and anti-inflammatory effect, making them natural and effective anticancer agents in a balanced diet [38]. The phenolic fraction of olive oil consists of a heterogeneous mixture of compounds, all of which influence the chemical properties and quality of the oil [15, 59]. International standards for the phenolic content of olive oil range from 50 to 1000 mg/kg, depending on several factors: variety, degree of ripeness, edaphic-climatic conditions, and cultivation methods [60–63]. Several oils studied have a total polyphenol content > 500 mg/kg, especially those coming from trees grown in the dry and harsh climatic conditions of the highlands, as environmental stress conditions activate the biosynthetic pathway for the accumulation of polyphenols [64]. The composition of phenols was also interesting, with tyrosols in sample M1 reaching 55.7 mg/kg, and hydroxytyrosol in M12 reaching 34.36 mg/kg, making these oils very interesting from a nutritional point of view. The oil "M11" was particularly rich in oleocanthal, oleuropein, oleuropein aglycone, and ligustroside aglycone, phenols that play an important role as chemopreventive compounds [65].

The fatty acid profile of olive oil is strongly influenced by the cultivar, fruit maturity, pedoclimatic growing conditions, and other minor area-related parameters [66]. Oleic acid was the most abundant, followed by palmitic acid, linoleic acid, and palmitoleic acid, with levels in agreement with the limits of the international food standards (23, 43) and in line with those of other local cultivars [29, 47], but significantly better than those of the most commonly grown cultivar "Chemlali", which has a palmitic acid content of > 21% and an oleic acid content of < 60% [67–70], suggesting that this germplasm contains variants for fatty acid components that could be used to improve commercial Tunisian cultivars.

Among PUFAs, the most abundant was linoleic acid, which cannot be synthesized by humans and must be supplied through the diet [65]. Nine oils, derived from ecotypes M5, M6, M8, M9, M10, M11, M14, M15, and M21, had an oleic acid/linoleic acid ratio > 7, the threshold value considered to ensure high quality and long shelf-life of the oil [47, 48, 71]. Previous studies have shown the key role of the enzyme fatty acid desaturase 2 (FAD2) in the oleic acid/linoleic acid profile, and how SNPs in the gene can influence

Table 4 Fatty acid composition of the studied millennium olive oils

Cultivar	Palmitic acid C16:0	Palmitoleic acid C16:1	Heptadecanoic acid C17:0	Heptadecanoic acid C17:1	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2	Linolenic acid C18:3	Arachidic acid C20:0	Gadoleic acid C20:1	C18:1/C18:2	∑SFA	∑MUFA
M1	15.50 ± 0.13 kl	1.65 ± 0.03jk	0.02 ± 0.00a	0.02 ± 0.00a	1.90 ± 0.13ab	68.66 ± 2.11gh	11.36 ± 0.42a	0.10 ± 0.00a	0.02 ± 0.00a	0.01 ± 0.00a	6.04 ± 0.17e	17.44 ± 0.27f	70.36 ± 2.15l
M2	8.00 ± 0.13 a	0.80 ± 0.00cd	0.09 ± 0.00a	0.12 ± 0.01a	2.98 ± 0.08h	75.00 ± 0.13lm	13.00 ± 0.06ab	0.40 ± 0.01bc	0.31 ± 0.08c	0.01 ± 0.00a	5.76 ± 0.02d	11.38 ± 0.30a	69 ± 0.15k
M3	15.30 ± 0.13jk	2.30 ± 0.01op	0.04 ± 0.00a	0.11 ± 0.00a	1.85 ± 0.03	65.20 ± 0.66e	13.93 ± 0.08b	0.34 ± 0.08b	0.12 ± 0.00ab	0.02 ± 0.00a	4.68 ± 0.00c	17.31 ± 0.17f	67 ± 0.69i
M4	13.06 ± 0.09ef	0.92 ± 0.01de	0.03 ± 0.00a	0.03 ± 0.00a	4.00 ± 0.2j	62.20 ± 0.4d	19.00 ± 0.13ab	0.40 ± 0.01bc	0.13 ± 0.03ab	0.01 ± 0.00a	3.27 ± 0.27b	17.22 ± 0.32f	63.16 ± 0.4f
M5	16.50 ± 0.2lm	1.89 ± 0.04lm	0.06 ± 0.00a	0.11 ± 0.01a	3.00 ± 0.08h	73.00 ± 0.33kl	5.05 ± 0.04a	0.42 ± 0.05cd	0.13 ± 0.00ab	0.03 ± 0.03a	14.45 ± 0.02i	19.69 ± 0.28h	75.03 ± 0.38p
M6	16.29 ± 0.27lm	1.80 ± 0.04kl	0.08 ± 0.00a	0.08 ± 0.00a	2.85 ± 0.01gh	73.15 ± 0.23kl	5.28 ± 0.27a	0.42 ± 0.01cd	0.15 ± 0.02ab	0.05 ± 0.06ab	13.85 ± 0.05h	19.38 ± 0.20h	75.08 ± 0.29p
M7	15.50 ± 0.26kl	0.65 ± 0.03ab	0.15 ± 0.2b	0.19 ± 0.33a	1.96 ± 0.07ab	68.00 ± 0.33gh	13.00 ± 0.06ab	0.80 ± 0.01kl	0.18 ± 0.00b	0.23 ± 0.01ef	5.23 ± 0.02d	17.79 ± 0.34f	77.24 ± 0.33r
M8	9.52 ± 0.39b	0.51 ± 0.01a	0.05 ± 0.00a	0.07 ± 0.00a	2.46 ± 0.11de	78.07 ± 0.38n	7.91 ± 0.11a	0.51 ± 0.04de	0.54 ± 0.02i	0.33 ± 0.01fg	9.86 ± 0.66h	12.57 ± 0.34b	72.8 ± 0.21n
M9	14.18 ± 0.39gh	1.33 ± 0.005hi	0.04 ± 0.00a	0.09 ± 0.00a	2.39 ± 0.13de	71.18 ± 0.2jk	9.88 ± 0.00a	0.55 ± 0.06ef	0.39 ± 0.08g	0.18 ± 0.00de	7.2 ± 0.01f	17.00 ± 0.23f	72.24 ± 0.22n
M10	14.60 ± 0.0hi	2.13 ± 0.22no	0.07 ± 0.00a	0.07 ± 0.00a	2.63 ± 0.11fg	70.00 ± 0.00ij	9.70 ± 0.00a	0.60 ± 0.00gh	0.36 ± 0.04ef	0.04 ± 0.00a	7.21 ± 0.16f	17.67 ± 0.13f	77.62 ± 0.24r
M11	10.60 ± 0.26bc	0.90 ± 0.0de	0.05 ± 0.00a	0.07 ± 0.00a	2.53 ± 0.02ef	76.33 ± 0.24mn	8.80 ± 0.2a	0.81 ± 0.02kl	0.32 ± 0.00c	0.32 ± 0.00a	8.67 ± 0.02g	13.50 ± 0.28c	68.85 ± 0.22j
M12	13.20 ± 0.06	1.08 ± 0.0ef	0.11 ± 0.00b	0.11 ± 0.00a	2.95 ± 0.00gh	67.53 ± 0.22fg	14.10 ± 0.13ab	0.69 ± 0.40hi	0.09 ± 0.00ab	0.13 ± 0.00cd	4.78 ± 0.02c	16.35 ± 0.06d	59.03 ± 0.65b
M13	15.10 ± 0.59ij	0.78 ± 0.02bc	0.08 ± 0.00a	0.12 ± 0.00a	1.77 ± 0.046a	58.00 ± 0.66b	22.74 ± 0.035	0.93 ± 0.00l	0.32 ± 0.02c	0.13 ± 0.00bc	2.55 ± 0.02a	17.27 ± 0.62f	71.87 ± 058m
M14	14.53 ± 0.37hi	1.10 ± 0.08fg	0.04 ± 0.00a	0.60 ± 0.006c	2.69 ± 0.01gh	69.90 ± 0.6ij	9.46 ± 0.24a	0.91 ± 0.01l	0.48 ± 0.00hi	0.26 ± 0.00ef	7.38 ± 0.00f	17.74 ± 0.37f	72.84 ± 0.33n
M15	14.53 ± 1.02hi	1.33 ± 0.04hi	0.03 ± 0.00a	0.06 ± 0.00a	2.53 ± 0.15ef	71.16 ± 0.28jk	9.01 ± 0.52	0.54 ± 0.09de	0.50 ± 0.00hi	0.28 ± 0.00ef	7.89 ± 0.00f	17.59 ± 0.93f	71.18 ± 0.24m
M16	13.39 ± 0.26fg	0.88 ± 0.06de	0.03 ± 0.00a	0.03 ± 0.00a	2.84 ± 0.01gh	70.00 ± 0.33ij	11.24 ± 0.29a	0.83 ± 0.01kl	0.46 ± 0.02hi	0.27 ± 0.15ef	6.22 ± 0.19e	16.72 ± 0.27d	62.67 ± 0.18e
M17	15.53 ± 0.28kl	0.93 ± 0.03de	0.04 ± 0.00a	0.06 ± 0.00a	1.85 ± 0.01ab	61.26 ± 0.17cd	18.65 ± 0.04ab	0.83 ± 0.00kl	0.45 ± 0.02hi	0.41 ± 0.01g	3.28 ± 0.06b	17.87 ± 0.24f	63.54 ± 0.024f
M18	15.48 ± 0.05kl	1.02 ± 0.04ef	0.04 ± 0.00a	0.05 ± 0.00a	1.79 ± 0.07a	62.22 ± 0.03d	18.10 ± 0.13ab	0.60 ± 0.01gh	0.42 ± 0.01gh	0.15 ± 0.05ef	3.43 ± 0.02b	17.73 ± 0.14f	50.28 ± 0.05g
M19	16.73 ± 0.08lm	2.71 ± 0.00r	0.04 ± 0.00a	0.06 ± 0.00a	2.56 ± 0.00ef	62.56 ± 0.04d	14.15 ± 0.03ab	0.72 ± 0.01jk	0.46 ± 0.00hi	0.27 ± 0.00ef	3.43 ± 0.03b	19.79 ± 0.09h	66.52 ± 0.5h
M20	14.76 ± 0.11hi	1.06 ± 0.00ef	0.03 ± 0.00a	0.03 ± 0.00a	4.01 ± 0.01j	65.16 ± 0.51e	14.73 ± 0.33ab	0.82 ± 0.01kl	0.57 ± 0.00i	0.26 ± 0.00ef	4.42 ± 0.02c	19.37 ± 0.09h	72.53 ± 0.05n
M21	15.73 ± 0.15kl	2.11 ± 0.01no	0.06 ± 0.00a	0.12 ± 0.00a	2.16 ± 0.02c	70.03 ± 0.04ij	9.26 ± 0.01a	0.71 ± 0.00ij	0.42 ± 0.01gh	0.26 ± 0.01ef	7.27 ± 0.24f	18.37 ± 0.11g	72.74 ± 0.08n
M22	11.40 ± 0.06cd	0.57 ± 0.00ab	0.06 ± 0.00a	0.05 ± 0.00a	2.51 ± 0.17ef	71.86 ± 0.08jk	12.51 ± 0.01ab	0.64 ± 0.00gh	0.41 ± 0.00g	0.25 ± 0.00ef	5.74 ± 0.45d	14.38 ± 0.05d	54.30 ± 0.08a
M23	19.73 ± 0.15n	2.60 ± 0.06qr	0.17 ± 0.00b	0.04 ± 0.00a	3.38 ± 0.022i	51.40 ± 0.13a	22.35 ± 0.03ab	0.85 ± 0.01kl	0.48 ± 0.01hi	0.25 ± 0.00ef	2.29 ± 0.19a	23.76 ± 0.12k	70.32 ± 0.23k
M24	11.96 ± 0.17de	0.92 ± 0.01de	0.05 ± 0.00a	0.10 ± 0.00a	3.88 ± 0.02j	69.16 ± 0.22hi	13.10 ± 0.43ab	0.57 ± 0.00fg	0.35 ± 0.00de	0.13 ± 0.00bc	5.27 ± 0.19d	16.24 ± 0.2^{e}	71.19 ± 0.06m
M25	15.03 ± 0.11ij	1.44 ± 0.00ij	0.16 ± 0.00b	0.11 ± 0.00a	1.94 ± 0.00ab	69.50 ± 0.06hi	11.29 ± 0.17	0.91 ± 0.00l	0.11 ± 0.0lab	0.13 ± 0.00bc	6.15 ± 0.01e	17.24 ± 0.11f	69.32 ± 0.17k
M26	16.53 ± 0.11lm	1.99 ± 0.00lm	0.15 ± 0.02b	0.04 ± 0.00a	1.77 ± 0.004a	67.26 ± 0.17ef	11.78 ± 0.18a	0.34 ± 0.006b	0.06 ± 0.00ab	0.03 ± 0.00a	5.7 ± 0.02d	18.51 ± 0.10g	74.92 ± 0.011o
M27	18.67 ± 0.17n	2.39 ± 0.00pq	0.03 ± 0.00a	0.06 ± 0.00a	2.17 ± 0.01cd	58.96 ± 0.04b	17.16 ± 0.11ab	0.11 ± 0.00a	0.13 ± 0.00ab	0.24 ± 0.01ef	3.43 ± 0.01b	21.00 ± 0.03j	61.66 ± 0.04d
M28	17.33 ± 0.04m	1.21 ± 0.00gh	0.10 ± 0.00a	0.34 ± 0.22b	1.92 ± 0.00ab	59.36 ± 0.04bc	20.53 ± 0.11ab	0.12 ± 0.00a	0.33 ± 0.01cd	0.02 ± 0.00a	2.89 ± 0.06a	19.68 ± 0.05h	60.93 ± 0.24c
Average	14.6	1.39	0.07	0.10	2.55	67.36	13.11	0.59	0.36	0.72	6.01	17.58	68.36

FAD2 activity [72, 73]. It will be very interesting to analyze the FAD2 sequence of these nine millennial olive ecotypes to further investigate the role of the FAD2 gene and include them in breeding programs to improve the quality of Tunisian EVOOs.

Conclusion

The compositional profile of the oils obtained from the millennial olive trees surviving in Tunisia makes them very interesting from a nutritional point of view, as beneficial properties are widely attributed to these compounds [43]. These trees have survived thousands of years in harsh conditions and are still productive, proving resilient to climate change, disease, and water scarcity, indicating careful selection by ancestors to meet their needs: cooking, lighting, and healing, which explains the high quality of the oil from these historic olive trees. This heritage is a treasure to be preserved and valorized for the development of the Tunisian olive sector, by including them in conservation and breeding programs to protect the environment and promote sustainable production. Future work will include accurate genotyping and authentication of the oil according to the consolidated protocols based on microsatellite markers for effective protection and valorization [74, 75]. We propose to recognize and protect these trees as living monuments and elements of the "biocultural heritage" and to preserve them in the arboretum of the Tunisian national Gene bank.

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Data availability Data are available at the link https://www.genesys-pgr.org/a/overview.

Declarations

Conflict of interest The authors declare they do not have competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Compliance with ethics requirements In relation to the Paper "Exploring the quality and nutritional profiles of monovarietal oils from millennial olive trees in Tunisia" published on the Journal European Food Research and Technology, We declare that this article does not contain any studies with human or animal subjects.

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