# Leaf morpho-physiology and phytochemistry of olive trees as affected by cultivar type and increasing aridity postprint

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## Abstract

The olive species (Olea europaea L.) is an ancient traditional crop grown under rainfed conditions in the Mediterranean Basin. In response to growing national and international demand for olive oil, olive cultivars are being introduced into highly arid new bioclimatic areas. Consequently, the morpho-physiology and phytochemistry of olive trees are potentially changing among cultivar types and geographical conditions. In the present work, we undertook an assessment of the impacts of geographical location and cultivar types on the leaf morpho-physiology and phytochemistry of olive trees.

## Full Text

## Preamble

**Leaf Morpho-Physiology and Phytochemistry of Olive Trees as Affected by Cultivar Type and Increasing Aridity**

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## Abstract

The olive species (*Olea europaea* L.) is an ancient traditional crop grown under rainfed conditions in the Mediterranean Basin. In response to growing national and international demand for olive oil, olive cultivars are being introduced into highly arid new bioclimatic areas. Consequently, the morpho-physiology and phytochemistry of olive trees are potentially changing among cultivar types and geographical conditions. In the present work, we undertook an assessment of the impacts of geographical location and cultivar types on the leaf morpho-physiology and phytochemistry of olive trees.

Leaves of the two most cultivated olive tree varieties, Chemlal and Sigoise, were collected from three geographical regions (Setif, Batna, and Eloued) with increasing aridity in Algeria. Leaf samples from the geographical regions were analyzed using standard physiological experiments, colorimetric methods, and chromatography assays. Leaves of both cultivars exhibited significant variance in terms of leaf shape index but not for leaf tissue density, specific leaf weight, and specific leaf area. Photosynthetic pigment contents were affected by both cultivar type and geographical location, with the lowest pigment content recorded in the Sigoise cultivar from the Setif region. Compared with the Setif and Batna regions, dried leaves of both cultivars from the Eloued region showed higher levels of total polyphenols, total flavonoids, and total tannins, as well as better antioxidant capacity. Liquid chromatography-mass spectrometry analysis of all leaf extracts identified the following phenolic acids as major compounds: oleuropein, naringin, apigenin-7-O-glucoside, kaempferol, quercetin, quercitrin, luteolin-7-O-naringenin, and quinic acid. Lower contents were found for p-coumaric acid, trans-ferulic acid, hyperoside, rutin, apigenin, caffeic acid, protocatechuic acid, o-coumaric acid, and gallic acid. Additionally, epicatechin and catechin+ were not found in the leaf extracts of the Sigoise cultivar. The leaf organic extracts from both cultivars displayed promising anti-cancer activity that was affected by geographical location and organic solvent polarity. Briefly, although increasing aridity and soil organic and mineral deficiency affected leaf morpho-physiological parameters, both cultivars sustained chemical richness, good antioxidant capacity, and anti-tumoral activity in leaves. Furthermore, the findings revealed that regardless of olive tree genotype, geographical location had a significant impact on leaf morpho-physiology, bioactivity, and chemical composition, which may consequently modulate the growth and oil production of olive trees.

**Keywords:** *Olea europaea* L.; aridity; leaf morpho-physiology; bioactivity; olive cultivar; geographical location; Algeria

## 1 Introduction

The olive tree (*Olea europaea* L.) has had a wide geographical distribution in Mediterranean regions for thousands of years (Dhia et al., 2018). According to Gomes et al. (2012), there are more than 8.05$×10^{8}$ olive trees in the world, 98.0% of which are distributed in Mediterranean regions. The global olive genetic heritage is characterized by a diversity of taxa (Muzzalupo et al., 2014) and includes about 30 genera and 600 species; particularly, the genus *Olea* L. includes more than 30 species distributed across Europe, Asia, Oceania, and Africa (Kermanshah et al., 2020).

In Algeria, olive trees are mainly cultivated in the northern mountainous part of the country (Abdessemed et al., 2018). Among the 36 identified olive cultivars in Algeria, Chemlal and Sigoise are the most cultivated (Ilarioni and Proietti, 2014), occupying 40.0% and 25.0% of olive orchards in Algeria, respectively (Douzane et al., 2021). Recently, a national program has been implemented to boost the development of intensive olive tree growing in the steppe, pre-Saharan, and Saharan areas in Algeria (including M'sila, Biskra, Ghardaïa, Batna, and Eloued). The launched sectoral development plan aims to increase the production of olive trees and olive oil, and to improve economic and ecological conditions in the more marginalized parts of the country. Because of the olive tree's exceptional longevity and aerial part with evergreen leaves and root system, it can preserve and protect soils from erosion and desertification in arid areas (Aïachi Mezghani et al., 2021). Given that olive oil has undeniable health benefits, increasing interest is being dedicated to the potential of olive leaves as a source of nutraceuticals and medicinal compounds for the prevention and treatment of several diseases in Mediterranean countries (Salah et al., 2012; Medina et al., 2019; Acar-Tek and Ağagündüz, 2020). Recent studies have shown that olive leaves contain many biologically active compounds (Markhali et al., 2020); for example, oleuropein is considered to promote health (Hassen et al., 2015), as it is associated with antispasmodic properties, immunomodulatory effects, and cardioprotective stimulants. It is also considered hypotensive and hyperdiabetic, and has anti-tumorous, anti-inflammatory, antioxidant, and anticoagulant functions. In addition, olive leaves contain triterpenic compounds such as oleanolic acid, which exhibit effective protective potential against various hepatotoxicants that can induce oxidative and electrophilic stresses (Guinda et al., 2015). Olive leaves are also being exploited as an alternative material for synthetic isotopes and fossil fuels (Gilani and Khan, 2010). The study of de Bock et al. (2013) showed that olive leaf polyphenol supply for 12 weeks significantly improved insulin sensitivity and pancreatic $\beta$-cell secretory capacity in overweight middle-aged males.

However, there have been reports that the chemical composition and bioactivity of olive trees are changing in response to seasonal variation (Wang et al., 2019), cultivar type, sampling time (Martín-García et al., 2019), and genetic diversity (Bilgin and Şahin, 2013; Talhaoui et al., 2015). Accordingly, it is of paramount importance to characterize the potential changes in leaf morpho-physiology and

phytochemistry of olive trees concurrently by considering cultivar diversity and the vast geographical distribution of olive trees in Algeria. This study can highlight the potential impact of geographical location on leaf morpho-physiology and phytochemistry with increasing aridity. Additionally, obtained data were analyzed to investigate whether the observed effects of aridity are cultivar dependent.

## 2 Materials and Methods

### 2.1 Study Area

The current research work was performed in three geographical regions with increasing aridity in Algeria (Setif, Batna, and Eloued). The Setif region $(36°23\$‘01‘‘N, 04°59’07‘‘E) has a semi-arid temperate climate with a mean annual precipitation of 583 mm, a mean$ has a Saharan climate with the lowest mean relative humidity of 30.0% and a mean annual precipitation of 65 mm (Fig. 1).

From the north (Setif region) to the south (Eloued region), there is a noticeable increase in the annual mean temperature against a decrease in annual precipitation, meaning that aridity gradually increases with distance from the ocean. Indeed, the annual mean temperature in the Eloued region is about two times higher than that in the Setif region. On the other hand, the annual precipitation in the Setif region is about nine times greater than that recorded in the Eloued region. As a result, the Batna region presents moderate values in annual mean temperature and precipitation (Fig. 1).

### 2.2 Experimental Design and Plant Materials

Leaves of two olive cultivars (Chemlal and Sigoise) were collected from three geographical regions of Eloued, Setif, and Batna with different aridity degrees. For the three regions, olive trees were planted in parallel with a spacing of 6.0 m$\$\times\$$6.0 m and tree ages ranged from 15 to 18 years. Both olive cultivars were subjected to drip system irrigation without any nutrient fertilization. Fresh leaves were collected from different sides of six trees in each cultivar’s land in each region in March 2019, with a total of 36 olive trees in the three geographical regions (Eloued, Setif, and Batna). A portion of the collected leaves was shade-dried, ground into a fine powder, and then stored in the dark until use.

### 2.3 Soil Sampling and Analysis

Soil samples were collected from the three geographical regions: Setif, Batna, and Eloued. Specifically, soil samples of each cultivar’s land in each region were taken twice from four soil layers (0–20, 20–40, 40–60, and 60–80 cm) with a helical auger. Soil samples were air-dried first. The samples from the same soil layer of the same cultivar land in each region were mixed to obtain a homogeneous medium specimen of fine soil and then sieved through a 2-mm sieve before analysis. Soil pH (HI2211-02, Hanna Instruments™ Woonsocket, Rhode

Island, USA) and electrical conductivity (EC) (Inolab, Cond 7310, Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Weilheim, Germany) were determined in a 1:5 soil:water suspension. Calcium carbonate content was measured by the calcimeter method (Nelson, 1983). Soil organic carbon (SOC) was determined using the mixture of $K_2Cr_2O_7$ and $H_2SO_4$ according to the method of Walkley and Black (1934), as reported in Nelson and Sommers (1983). The distribution of soil particle sizes was determined using the method of Robinson (1922).

### 2.4 Leaf Morpho-Physiology

Leaf shape was determined by the value of the leaf shape index, which is the ratio of leaf length (LL; cm) to leaf width (LW; cm) (IOC, 1997). The leaf shape is elliptical if the leaf shape index is lower than 4, elliptical-lanceolate if the leaf shape index is higher than 4 but lower than 6, and lanceolate if the leaf shape index is higher than 6. Leaf length and width were measured using graph paper.

According to Shaheen et al. (2011), the average leaf area (LA; $cm^2$) was determined with the following equation:

$$LA = 0.53 \times LW \times LL + 1.66$$

The leaf tissue density (LTD; mg/mg) was calculated using the following equation (Bacelar et al., 2006):

$$LTD = \frac{DW}{FW} \times 1000$$

where DW is the dry weight (mg) and FW is the fresh weight (mg).

The specific leaf area (SLA; $cm^2$/mg) was determined using Equation 3 (Datt B, 1999):

$$SLA = \frac{LA}{DW}$$

The specific leaf weight (SLW; mg/$cm^2$) was calculated by Equation 4 (Datt B, 1999):

$$SLW = \frac{DW}{LA}$$

### 2.5 Chlorophyll and Carotenoid Contents

For each cultivar, three fresh leaves were taken from the middle part of the olive tree, and then a disc of 50.0 mg from the central part of the fresh leaf was used. Each leaf sample was ground with 10.0 mL of methanol (99.0%) and a small amount of calcium carbonate, and then placed in a tightly closed tube. The leaf samples were left for 48 h in the dark at 4.0°C. After that, the methanolic extract was filtered (Lichtenthaler and Buschmann, 2001), and a spectrophotometer (Jenway 6300, Jenway® Equipment for Analysis, Staffordshire, UK) was used to read the absorbance (A; nm) of the samples at wavelengths of 665.2, 652.4, and 470.0 nm. The following equations were used to estimate chlorophyll and carotenoid contents:

$$Chlorophyll\ a = 16.7 \times A_{665.2} - 8.36 \times A_{652.4}$$

$$Chlorophyll\ b = 29.16 \times A_{652.4} - 4.96 \times A_{665.2}$$

$$Chlorophyll\ a + b = 24.93 \times A_{652.4} + 4.24 \times A_{665.2}$$

$$Carotenoid = \frac{1000 \times A_{470} - 1.63 \times Chl_a - 104.96 \times Chl_b}{221}$$

where chlorophyll a, chlorophyll b, chlorophyll a+b, and carotenoid contents were given in µg/mL and then converted into µg/g FW.

### 2.6 Phytochemical Analysis

**2.6.1 Metabolite Extraction** Fractionated extraction was performed using three organic solvents of increasing polarity (hexane, dichloromethane, and methanol). After evaporation of the organic solvents using a rotary evaporator, the dry residue from each extraction was weighed and stored at –20.0°C.

**2.6.2 Determination of Total Phenolic Content** Total phenolic content was determined using the Folin-Ciocalteau reagent according to the method of Singleton and Rossi (1965). The Folin-Ciocalteau reagent is reduced by polyphenols to tungsten oxide with blue color. The resulting color has maximum absorption intensity at a wavelength of 765.0 nm, which is proportional to the total phenolic content in the extraction (Georgé et al., 2005). Exactly 100.0 µL of the methanol leaf extract solution at 1.00 mg/mL was taken, and 500.0 µL of 10-times diluted Folin-Ciocalteau reagent was added. The tubes were shaken for 3 min, and then 400.0 µL of sodium carbonate solution $Na_2CO_3$ (7.5%) was added. The tubes were shaken well and incubated at laboratory temperature away from light for 30 min. The absorbance of the various samples was measured

in a spectrophotometer at a wavelength of 765.0 nm. The result was expressed in milligram gallic acid equivalent per gram extract (mg GAE/g DW).

**2.6.3 Determination of Total Flavonoid Content** Total flavonoid content was determined using aluminum trichloride and sodium hydroxide. Aluminum trichloride ($AlCl_3$) forms a yellow compound with flavonoids, and the soda forms a pink compound that can be absorbed in the visible range at 510.0 nm (Zhishen et al., 1999). The leaf extract diluted with 1.0 mL methanol was added to 1.0 mL of $AlCl_3$ (2.0% methanol solution), then the tubes were shaken and incubated at laboratory temperature in the dark for 10 min. The absorbance was read in a spectrophotometer at a wavelength of 430.0 nm. The result was expressed as milligram quercetin equivalent per gram extract (mg QE/g DW).

**2.6.4 Determination of Tannin Content** The quantitative tannin content of leaf extracts was determined using the method of Schofield et al. (2001). The principle is based on the fixation of the aldehyde group of vanillin on the C6 carbon of the catechin A-ring to form a red compound (chloroform), which is absorbed at a wavelength of 500.0 nm. Exactly 400.0 µL of the olive leaf extract was added with 3.0 mL of vanillin solution (4.0%) and 1.5 mL of concentrated hydrochloric acid. The mixture was incubated for 15 min and the absorbance was measured at a wavelength of 500.0 nm. The result was expressed as milligram catechin equivalent per gram extract (mg CE/g DW).

**2.6.5 Secondary Metabolite Analysis Using Liquid Chromatography Coupled to Mass Spectrometry** Liquid chromatography-mass spectrometry was used to identify phenolic compounds in our samples. LC-MS analysis of the compounds was performed with the Shimadzu LC-20ADXR instrument (Shimadzu, Kyoto, Japan) equipped with a SIL-20AXR autosampler (40.0°C). Separations were performed at 75.0°C in a GLScience C18 column (length of 150 mm, inner diameter of 3 mm, and particle size of 3 µm). The column was run at a flow rate of 0.4 mL/min, and the mobile phase was combined with acidified MeOH/$H_2O$ (5%:95% acetic acid at 0.15%; eluent A) and acetonitrile/water (50%:50% acetic acid at 0.15%; eluent B). The gradient program was used as follows: 0–14 min with 10.0%–20.0% eluent B, 10–27 min with 20.0% eluent B, 27–37 min with 20.0%–55.0% eluent B, 37–45 min with 55.0% eluent B, and finally 45–52 min with 55.0%–100.0% eluent B. Detection was performed by a Shimadzu LC-MS 2020 single quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source. Identification of compounds was confirmed by comparison relative to standard molecules.

### 2.7 Antioxidant Activity

The antioxidant activity of olive leaf extracts was assayed in vitro against 2,2-diphenyl-1-picrylhydrazyl (DPPH). The DPPH radical scavenging antioxidant activity of methanol extracts of olive leaves was assayed using the method de-

scribed by Benhammou et al. (2009). A volume of 500.0 µL of different concentrations (0.01, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, and 0.40 mg/mL) from each extract was added to 500.0 µL of 0.25 mM DPPH. Two replicates were used for all preparations, and then the samples were shaken and kept in the dark at laboratory temperature for 30 min. The absorbance was measured at a wavelength of 517.0 nm. Ascorbic acid was used as a standard in inhibiting free radicals. The percentage of DPPH inhibition (I; %) was calculated for the different concentrations of olive leaf extracts using the following equation:

$$I = \frac{A_0 - A_I}{A_0} \times 100$$

where $A_0$ is the absorbance of the control sample (nm) and A  is the absorbance of the leaf sample (nm). Antioxidant activity was estimated by calculating the half-maximal inhibitory concentration ($IC_{50}$; µg/mL), which is defined as the concentration of extract required to inhibit 50.0% of the DPPH radical.

### 2.8 Cytotoxic Activity

The cytotoxic effect of samples was evaluated using Human Colon Cancer (HCT116) and Colorectal Carcinoma (CaCo-2) cell lines, ordered from the American Type Culture Collection (ATCC Co., Manassas, USA), as described by Bekir et al. (2013) with modifications (Rahmani et al., 2019). Cells were cultured in high glucose Roswell Park Memorial Institute (RPMI) and Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Les Ulis, France), supplemented with 10.0% fetal bovine serum and 1.0% penicillin-streptomycin and gentamicin in a humidified atmosphere with 5.0% $CO_2$ at 37°C, respectively. The cell suspension was distributed into 96-well plates at 13$×10^{3}$ and 12$×10^{3}$ cells/well in 100.0 µL, and then treated with 100.0 µL of sample dilution prepared with corresponding culture medium at concentrations of 50.0 and 5.0 mg/L, respectively. The samples were solubilized in Dimethyl sulfoxide at 5.00 mg/mL concentration. Cell growth was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT test is a colorimetric test that measures the reduction of yellow MTT bromide to insoluble dark purple formazan crystals by mitochondrial succinate dehydrogenase of living cells. The solubilized formazan reagent is released by Dimethyl sulfoxide and measured by spectrophotometry at a wavelength of 605.0 nm. Tamoxifen was used as a positive control. The cell activity inhibition percentage was calculated as:

$$Activity\ inhibition\ percentage = \frac{A_{sample} - A_{blank}}{A_{blank}} \times 100$$

where A_{blank} is the absorbance of the assay medium and MTT solution without olive tree leaf extract (nm), and A_{sample} is the absorbance of the assay medium and MTT solution added with olive tree leaf extract (nm).

### 2.9 Statistical Analysis

Data were studied using analysis of variance (ANOVA). Mean averages were compared by Fisher's LSD test and considered significant at $P<0.05$ level. Statistical analyses were computed using Minitab 17 statistical software. Principal component analysis (PCA), based on Pearson's product-moment correlation at $P<0.05$ level, was performed separately on morpho-physiological and phytochemical parameters. The study was conducted using XLSTAT software (version 2009.4.03).

## 3 Results

### 3.1 Bioclimatic and Pedological Conditions

As shown in Table 1 , soil pH values of the three geographical regions ranged from 7.53–8.39, indicating alkaline soil. These alkaline pH values were likely indicative of carbonate presence. Similar pH results were reported in soils of several olive groves in Tunisia (Omar et al., 2017). ANOVA results showed that geographical location and cultivar type ($P=0.000$) exhibited very significant impact on pH, but not an interaction between them ($P>0.050$; Table 1).

According to recorded low EC values (<1.59 mS/cm), all sampled soils were non-saline (Table 1). The obtained EC data were consistent with earlier findings reported by Zaanouni et al. (2018) for olive grove soils in Tunisia, where EC varied between 0.80 and 1.60 mS/cm. The lowest EC value was measured in soils under Chemlal olive trees in the Setif region, while the highest EC value was observed in soils under Chemlal olive trees in the Batna region. ANOVA results revealed a highly significant effect ($P=0.000$) of all factors on EC (Table 1).

SOC content, $CaCO_3$ content, and soil texture varied greatly between Sigoise and Chemlal cultivated lands (Table 1). For example, soils from the Eloued region displayed the lowest SOC contents of about 0.13% and 0.11% in Sigoise and Chemlal cultivated lands, respectively. However, soils from the Setif region showed maximum SOC content in Sigoise cultivated land. All studied soils were considered poor as SOC content did not exceed 2.00%. ANOVA results indicated that SOC was significantly affected by cultivar type, geographical location, and their interaction ($P<0.050$). High variability of $CaCO_3$ levels was observed among analyzed soils. Higher $CaCO_3$ contents were obtained in soils from Sigoise and Chemlal cultivated lands in the Batna region. According to $CaCO_3$ levels, all soils could be classified as carbonated. Textural analysis showed that soils from the Eloued region were mostly sandy for both Chemlal (95.7%) and Sigoise (94.7%) cultivated lands. Lower sand percentages were found in soils of Setif and Batna regions (Table 1). Soils from Setif and Batna regions exhibited fine texture with clay percentage of 31.0%.

In the current study, properties of soils from Setif and Batna regions were similar to those reported in previous studies on soils of semi-arid regions in Algeria

(Ababsa et al., 2020; Mehalaine and Chenchouni, 2020; Bona et al., 2021). Likewise, soils from the Eloued region showed the same characteristics as soils in arid regions of Tunisia (Omar et al., 2017; Brahim et al., 2021).

### 3.2 Leaf Morpho-Physiology

**3.2.1 Leaf Morphology** Analyzed morpho-physiological parameters shown in Table 2 indicated that both cultivars from different regions showed an elliptical-lanceolate pattern in leaf shape. However, there was slight difference in leaf shape index, with average leaf length ranging from 5.56 to 7.43 cm and average leaf width from 1.19 to 1.47 cm (Table 2). The largest values of leaf shape index were recorded as 5.52 ($\pm$0.46)*inChemlalcultivatedlandoftheSetifregionand*5.51($\pm$0.31)*inSigoisecultivatedlandoftheBatnaregion* These results were similar to those of Ozkaya et al. (2006), showing that leaf shape index of Derik Halhali olive leaf cultivar grown in Turkey ranged from 6.35 to 3.48. ANOVA results indicated that leaf shape index was more influenced by geographical location and the interaction between geographical location and cultivar type ($P<0.050$) than by cultivar type alone ($P>0.050$).

The Sigoise cultivar in the Setif region had the largest leaf area of 7.42 $cm^2$ (Table 2), while the Chemlal cultivar in the Eloued region had the lowest value of 5.15 $cm^2$. Similar results have been recorded in five olive tree cultivated lands in Portugal, with leaf area ranging from 3.51 to 7.69 $cm^2$ (Bacelar et al., 2004). ANOVA results showed extreme and highly significant differences in leaf area between geographical location and cultivar type ($P=0.000$), but the interaction between them did not change significantly. Increasing aridity was associated with decreased leaf area (Table 2). Numerous previous studies reported that olive tree cultivars grown in xeric areas exhibited smaller leaves than genotypes in mesic areas (Pita and Pardos, 2001; Bacelar et al., 2004), which may reduce water loss through transpiration.

**3.2.2 Leaf Sclerophylly Indices** The Chemlal cultivar of the Batna region recorded the highest specific leaf area (0.064 $cm^2$/mg), while the Chemlal cultivar of the Eloued region showed the lowest (0.039 $cm^2$/mg) (Table 2). ANOVA results revealed that specific leaf area was mostly affected by geographical location ($P=0.000$), which was also recorded for other Mediterranean cultivars (Guerfel et al., 2009; Ennajeh et al., 2010). Conversely, there were no significant effects of cultivar type or the interaction between geographical location and cultivar type on specific leaf area.

The Chemlal cultivar in the Eloued region recorded the highest average specific leaf weight at 25.46 mg/$cm^2$, while the Sigoise cultivar in the Batna region presented the lowest at 17.44 mg/$cm^2$ (Table 2). According to variance analysis, specific leaf weight was highly affected by geographical location ($P=0.000$) but not significantly changed by cultivar type ($P=0.130$) or the interaction between geographical location and cultivar type ($P=0.840$).

There was convergence in leaf tissue density of both Chemlal and Sigoise cultivars in the three geographical regions, which varied between 457.50 and 565.63 mg/g (Table 2). The leaf tissue density of the Chemlal cultivar in the Batna region recorded the lowest value among the two cultivars in all regions. Statistical analysis showed that leaf tissue density was significantly affected by geographical location (P=0.003), which was also reported in olive tree leaves of two Tunisian cultivars (Chemlali and Chétoui) growing under arid conditions (Guerfel et al., 2009). Leaf tissue density was not significantly regulated by cultivar type, suggesting that environment rather than genotype modulated this morphological parameter.

Based on leaf morphological analysis, it could be concluded that the two olive cultivars, Chemlal and Sigoise, belonged to the elliptical-lanceolate cultivar group in the growing areas (IOC, 1997). Regarding morpho-physiological characteristics, Connor (2005) confirmed that environmental conditions significantly affect properties of olive leaves during the growing season. Here, increasing aridity was associated with highly significant changes in leaf morpho-physiology of both cultivars (Table 2). Taken together, values of leaf morpho-physiological parameters decreased with increasing aridity, showing that leaf area reduced, small cell size decreased, and cell wall elasticity changed. It appears that smaller olive trees in the Eloued region were better adapted to water deficit and high transpiration conditions (Connor and Fereres, 2010). In fact, lower air vapor pressure could ensure less plant transpiration under high drought conditions (Tognetti et al., 2009; Diaz-Espejo et al., 2012). In addition to geographical location influence, cultivar type also affected leaf morpho-physiological parameters, among which the Chemlal cultivar displayed less effect under drought conditions (Table 2).

**3.2.3 Total Chlorophyll and Carotenoid Contents** Determination of total chlorophyll a+b revealed that in the Batna region, the highest content (1728.58 µg/g FW) was recorded for the Sigoise cultivar, while the lowest (864.37 µg/g FW) was found for the Chemlal cultivar (Table 2). Compared with the Setif region, total chlorophyll content of the Sigoise cultivar was reduced by 35.0% in the Eloued region, primarily due to a drop in chlorophyll a level (Table 2). However, for the Chemlal cultivar, leaf chlorophyll content did not change among different geographical regions. The achieved results in total chlorophyll content were similar to those reported in olive leaves of four Tunisian cultivars (Bahloul et al., 2014) and higher than those reported in olive trees of other Mediterranean countries (Maayan et al., 2008; Brahmi et al., 2012; Tekaya et al., 2016). Statistical analysis revealed that cultivar type was the prevalent factor influencing chlorophyll content (P=0.000), which was also argued by Bahloul et al. (2014).

Regardless of geographical location, higher carotenoid content in olive leaves was recorded in the Sigoise cultivar compared to the Chemlal cultivar. For both cultivars, increasing aridity was associated with improvement of total carotenoid

content (Table 2). In various higher plants, carotenoids were found to play a direct role in drought tolerance by reducing reactive oxygen species toxicity (Niyogi et al., 1997). Tolerant plant species, compared to sensitive genotypes, accumulated higher carotenoid contents under drought conditions (Parida et al., 2007). ANOVA results showed that carotenoid content was separately and significantly affected by cultivar type and geographical location but not by their interaction.

### 3.3 Phytochemical Composition

**3.3.1 Total Polyphenol and Flavonoid Contents** For both olive cultivars, the highest total polyphenol content was recorded in the Eloued region (Table 2). Observed changes in total phenolic content among harvesting locations could be attributed to arid climatic and environmental factors (Miliauskas et al., 2004; Gharibi et al., 2019). Sarker and Oba (2018) found that 16 phenolic acids and flavonoids in leaves of *Amaranthus tricolor* were remarkably increased with severity of drought stress. According to results in Table 2, changes in total phenolic content values in leaves of both olive cultivars could be partially linked to increment of total flavonoid content.

**3.3.2 Total Tannin Content** In plant species, tannins play a major role in defense against pathogens (Marsh et al., 2020) and contribute to protein binding (Jones and Palmer, 2000; Karonen et al., 2015) and antioxidant activity (Moilanen et al., 2016). According to Table 2, total tannin content in leaves of both cultivars varied from 2.14 to 3.45 mg CE/g DW. ANOVA results showed that total tannin content in leaves of the two olive cultivars was significantly affected by geographical location (P=0.016) but not by cultivar type (P=0.846).

**3.3.3 Identification of Phenolic Compounds in Olive Leaves** Liquid chromatography-mass spectrometry analysis on dry residue extracts of olive leaves from Sigoise and Chemlal cultivars enabled identification of 20 phenolic compounds (Tables 3 and 4 ). The obtained data revealed the richness of olive leaves in phenolic compounds, including oleuropein, luteolin-7-O-glucoside, kaempferol, quercetin, quercitrin, apigenin-7-O-glucoside, quinic acid, and naringenin in all geographical regions and with different solvents (Tables 3 and 4).

Oleuropein was the major phenolic compound (Tables 3 and 4), as also reported for other olive tree cultivars (Petridis et al., 2012; Zeitoun et al., 2017). The analyzed olive leaves showed average contents of other phenolic compounds such as p-coumaric acid, rutin, trans-ferulic acid, hyperoside, naringin, quercetin, quercitrin, and apigenin. Other compounds were very weakly identified: catechin+, epicatechin, caffeic acid, o-coumaric acid, gallic acid, and protocatechuic acid. The LC-MS data indicated that methanol extraction allowed detection of maximum phenolic compounds compared to other solvents (Tables 3 and 4). The highest oleuropein content was recorded in methanolic extract of olive leaves from the Sigoise cultivar in the Batna region at 15,900.86 mg/kg. The methano-

lic extract of the Chemlal cultivar from the Eloued region exhibited predominance in quinic acid, gallic acid, protocatechuic acid, luteolin-7-O-glucoside, naringin, o-coumaric acid, and quercetin; the same was found for the Sigoise cultivar from the Eloued region, namely with rutin, hyperoside, and quercitrin (Tables 3 and 4).

The methanolic extract of the Chemlal cultivar from the Setif region was relatively enriched in catechin+, trans-ferulic acid, and kaempferol, while the Sigoise cultivar was more enriched in apigenin. Dichloromethane extracts of olive leaves from the Batna region showed slight superiority in epicatechin and naringenin contents for the Chemlal cultivar and higher content of caffeic acid for the Sigoise cultivar (Tables 3 and 4).

Predominance of oleuropein and luteolin-7-O-glucoside as phenolic compounds in analyzed olive leaves was also reported in previous studies (Pereira et al., 2007; Mohamed et al., 2018). It should be emphasized that several factors can qualitatively and quantitatively modify olive leaf composition in terms of phenolic compounds (Bilgin and Şahin, 2013), such as olive tree variety as well as water deficit, salinity, fertilization, geographical location (Vinha et al., 2005), sample collection time, and climatic conditions (Talhaoui et al., 2015).

Detected phenolic compounds can be classified into three categories: (1) phenolic acids (quinic acid, gallic acid, protocatechuic acid, caffeic acid, p-coumaric acid, o-coumaric acid, and trans-ferulic acid); (2) oleuropeoside (oleuropein); and (3) flavonoids (flavan-3-ols (catechin+ and epicatechin), flavanones (naringenin and naringin), flavones (apigenin, luteolin-7-O-glucoside, and apigenin-7-O-glucoside), and flavonols (rutin, hyperoside, quercitrin, kaempferol, and quercetin)).

Differences in phytochemical composition contents in leaf extracts of the Chemlal cultivar in the three geographical regions are illustrated in Figure 2 [Figure 2: see original paper]. Leaf extracts of the Chemlal cultivar in the Eloued region were most enriched in all phenolic compounds except flavan-3-ols, which were not detected (Fig. 2). However, the lowest contents of detected phenolic compounds were recorded in olive leaves from the Setif region (Fig. 2). Divergent data have been reported regarding richness of olive leaves in phenolic compounds. These differences may be due to variety, temperature, drying duration, ripening, time of harvest (Al Juhaimi et al., 2018), and geographical location (di Donna et al., 2010). Additionally, Bilgin and Şahin (2013) reported that the phenolic profile of olive leaves is affected by several agronomic and technological factors such as leaf age, degree of maturity, geographical location, variety, phenological stage at sampling, proportion of branch on the tree, moisture content, degree of soil contamination, and industrial extraction processes (Ahmad-Qasem et al., 2013).

### 3.4 Antioxidant Activity

Olive leaf antioxidant activity was analyzed based on its scavenging capacity of free DPPH radical (Table 2 ). The $IC_{50}$ value was inversely related to antioxidant capacity of the extract, expressing the number of antioxidants needed to scavenge 50.0% of free DPPH radicals. Therefore, the lower the $IC_{50}$ value, the higher the antioxidant activity.

Antioxidant activity was slightly improved with increasing aridity for both cultivars (Table 2). The most potent antioxidant capacity was recorded in leaves of the Sigoise cultivar from the Eloued region and the Chemlal cultivar from the Batna and Eloued regions (Table 2). For both cultivars, the highest antioxidant activity of olive leaf extracts in the Batna and Eloued regions was correlated with higher contents of total polyphenols and flavonoids (Table 2) and oleuropein (Tables 3 and 4). Olive leaf extracts were an essential source of antioxidants, such as total phenolic compounds and flavonoids (Tables 3 and 4), which exhibited effective antioxidant activity (Condelli et al., 2015; Borjan et al., 2020; Martín-García et al., 2022). The high levels of oleuropein recorded in methanolic extracts of both cultivars (Tables 3 and 4) appeared to be the most crucial phenolic compound conferring antioxidant capacity to olive leaves (Lins et al., 2018). Statistical data showed that antioxidant capacity was concomitantly affected by both cultivar type and geographical location.

### 3.5 Cytotoxic Activity

Cytotoxic activity of organic extracts against HCT116 and Caco-2 cell lines was performed using the MTT assay, which is reliable for detecting cell proliferation. Cytotoxic activity of leaves from the Chemlal cultivar was affected by extraction solvent and geographical location (Fig. 3 [Figure 3: see original paper]).

The strongest cytotoxic activity against the HCT116 cell line was recorded in leaves of the Chemlal cultivar from the Batna region extracted with hexane, with inhibition reaching over 70.0% (Fig. 3a). Extracts with MeOH from the Chemlal cultivar displayed the weakest anti-HCT116 activity, with inhibition ranging from 20.0% to 29.0%. Organic extracts from the Sigoise cultivar showed lower activity against the HCT116 cell line, which did not exceed 50.0%.

Better activity of both cultivars was recorded against the Caco-2 cell line (Fig. 3b). Potent anti-Caco-2 activity of about 80.0% was measured in hexane extracts from the Chemlal cultivar in all geographical regions and from the Sigoise cultivar in the Batna region (Fig. 3b). Conversely, the weakest anti-Caco-2 activity was obtained for organic leaf extracts from the Sigoise cultivar in the Eloued region (Fig. 3b). Olive leaf extracts exhibited strong cytotoxic activity against various cancer cell lines (Antoniou and Hull, 2021), including human colon (Hashim et al., 2008; Corona et al., 2009; Terzuoli et al., 2016), pancreatic (Goldsmith et al., 2015), and leukemia (Abaza et al., 2007).

Accordingly, it could be concluded that hexane extracts of both olive cultivars

exhibited the highest anti-tumoral capacity relative to dichloromethane (DCM) and methanol (MeOH) ones. Conversely, the accumulative content of oleuropein was low in leaf hexane extracts (Tables 3 and 4). Therefore, anti-cancer capacity of leaf extracts from both cultivars could not be attributed only to oleuropein but also to other biomolecules, and may result from synergetic effects. Ruzzolini et al. (2018) suggested that olive leaf extract enriched in oleuropein could be more effective when other polyphenols are present. However, de Marino et al. (2014) found that in olive tree leaves, there were other compounds in addition to oleuropein, such as secoxyloganin and tyrosol, which have high anticancer potential. Statistically, there was significant difference in cytotoxic activity against Caco-2 among all factors, but anti-HCT116 activity was mostly affected by geographical location and solvent polarity, as well as their interaction (Table 5 ).

### 3.6 Data Statistical Analysis

Pearson's correlation matrix was used to analyze descriptors of morpho-physiology, photosynthetic pigment concentration, phenolic content, antioxidant, and cytotoxic activity of studied cultivars in different geographical regions to illustrate associations of individuals or links between variables (Table 6 ). Analysis revealed that cytotoxic activity against HCT116 cell lines exhibited positive correlations with flavanones and flavones, while cytotoxic activity against Caco-2 cell lines showed positive correlations with hydroxybenzoic and hydroxycinnamic acids (Table 6). Conversely, weak correlation was reported between anti-cancer activities (anti-HCT116 and Caco-2 cell lines) and contents of flavan-3-ols and flavonols (r=0.092 and r=0.034, respectively).

PCA analysis showed that two axes (PC1 and PC2, where PC is principal component) explained most of observed variability (62.84%), the first of which was most significant at 42.28% of total inertia (Fig. 4 [Figure 4: see original paper]). There was positive correlation between flavones and variables related to both cultivars in the Eloued region, which were particularly affected by leaf tissue density, specific leaf weight, cyclohexanecarboxylic acids, hydroxycinnamic acids, flavan-3-ols, total flavonoid content, flavonols, flavones, flavanones, and total phenolic content. Likewise, for Chemlal cultivar in the Batna region, variables included hydroxybenzoic acids, cytotoxic activities against HCT116 and Caco-2 cell lines, oleuropeoside, and total carotenoid content. The second axis accounted for 20.56% of total inertia and showed positive correlation for variables related to both cultivars in the Setif region, including chlorophyll b, leaf dry weight, leaf fresh weight, leaf shape index, leaf length, $IC_{50}$, and total carotenoid content.

The PC1 and PC2 axes of the two cultivars can be divided into four groups. The first comprised the Sigoise cultivar in the Eloued region, situated on the positive side of both PC1 and PC2 axes (Fig. 4). The second group comprised the Chemlal cultivar in the Eloued and Batna regions, placed on the negative side of PC1 axis and positive side of PC2 axis. The third group included both

cultivars in the Setif region, located on the positive side of PC1 axis and negative side of PC2 axis. The final group included the Sigoise cultivar in the Batna region, placed on the negative side of both PC1 and PC2 axes. PCA results also reported significant distinctions in morpho-physiological parameters, phenolic content, antioxidant activity, photosynthetic pigment content, and cytotoxic activity between the two cultivars according to geographical location (Fig. 4).

## 4 Conclusions

The Sigoise and Chemlal cultivars were growing on non-saline, alkaline, and carbonated soils. Both cultivars grown on poor sandy soils in arid climate in the Eloued region. Assessment of leaf morpho-physiological parameters in olive trees revealed that geographical location affected leaf shape index. Nevertheless, leaf tissue density, specific leaf weight, and leaf area were unchanged for both cultivars, which may sustain sufficient photosynthetic capacity among changes in climatic conditions associated with geographical location. Total phenolic content increased with aridity, and oleuropein was the most abundant phenolic acid in leaves of both cultivars. The highest oleuropein content in methanolic leaf extracts from Batna and Eloued regions was associated with better antioxidant capacity. However, the most robust cytotoxic activity against HCT116 and Caco-2 cell lines was unrelated to oleuropein richness in leaves of both cultivars. Statistical analysis showed that cytotoxic capacity was positively correlated with other bioactive compounds such as flavanones and hydroxybenzoic acids. All obtained results argued that introducing Sigoise and Chemlal cultivars in geographical locations with increasing aridity was associated with leaf morpho-physiological adaptations, phenolic acid and flavonoid enrichment, and improved leaf bioactivity.

Further experiments are suggested to (i) assess the impacts of combined effects of cultivar type and geographical location on growth and yield of olive trees and (ii) identify the active molecules regulating antioxidant and anti-cancer potentialities from crude leaf extracts.

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## References

Ababsa N, Kribaa M, Tamrabet L, et al. 2020. Long-term effects of wastewater reuse on hydro-physical characteristics of grassland grown soil in semi-arid Algeria. *Journal of King Saud University–Science*, 32(1): 1004–1013.

Abaza L, Talorete T P N, Yamada P, et al. 2007. Tunisian Gerboui olive leaf extract induces growth inhibition and differentiation of human leukemia HL-60

cells. *Journal Bioscience Biotechnology and Biochemistry*, 71(5): 1306–1312.

Abdessemed S, Abdessemed A, Boudchicha R H, et al. 2018. Characterization and identification of some olive ecotypes (*Olea europaea* L.) in Algeria. *Agriculture Journal*, 8(2): 26–43. (in French)

Acar-Tek N, Ağagündüz D. 2020. Olive leaf (*Olea europaea* L. folium): Potential effects on glycemia and lipidemia. *Annals of Nutrition and Metabolism*, 76(1): 10–15.

Ahmad-Qasem M H, Cánovas J, Barrajón-Catalán E, et al. 2013. Kinetic and compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound. *Innovative Food Science and Emerging Technologies*, 17: 120–129.

Aïachi Mezghani M, Laaribi I, Zouari I, et al. 2021. Sustainability and plasticity of the olive tree cultivation in arid conditions. In: Khebour Allouche F, Abu-hashim M, Negm A M. *Agriculture Productivity in Tunisia under Stressed Environment*. Springer Water. Cham: Springer, 27–56.

Al Juhaimi F, Özcan M M, Uslu N, et al. 2018. The effects of conventional heating on phenolic compounds and antioxidant activities of olive leaves. *Journal of Food Science and Technology*, 55: 4204–4211.

Antoniou C, Hull J. 2021. The Anti-cancer effect of *Olea europaea* L. products: a review. *Current Nutrition Reports*, 10(1): 1–15.

Bacelar E A, Correia C M, Moutinho-Pereira J M, et al. 2004. Sclerophylly and leaf anatomical traits of five field-grown olive cultivars growing under drought conditions. *Tree Physiology*, 24(2): 233–239.

Bacelar E A, Santos D L, Moutinho-Pereira J M, et al. 2006. Immediate responses and adaptive strategies of three olive cultivars under contrasting water availability regimes: Changes on structure and chemical composition of foliage and oxidative damage. *Plant Science*, 170(3): 596–605.

Bahloul N, Kechaou N, Mihoubi N B. 2014. Comparative investigation of minerals, chlorophylls contents, fatty acid composition and thermal profiles of olive leaves (*Olea europaea* L.) as by-product. *Grasas y Aceites*, 65(3): e035, doi: 10.3989/gya.0102141.

Bekir J, Mars M, Souchard J P, et al. 2013. Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (*Punica granatum*) leaves. *Food and Chemical Toxicology*, 55: 470–475.

Benhammou N, Bekkara F A, Panovska T K. 2009. Antioxidant activity of methanolic extracts and some bioactive compounds of *Atriplex halimus*. *Comptes Rendus Chimie*, 12(12): 1259–1266.

Bilgin M, Şahin S. 2013. Effects of geographical origin and extraction methods on total phenolic yield of olive tree (*Olea europaea*) leaves. *Journal of the Taiwan Institute of Chemical Engineers*, 44(1): 8–12.

Bona E, Massa N, Toumatia O, et al. 2021. Climatic zone and soil properties determine the biodiversity of soil bacterial communities associated with native plants from desert areas of North-Central Algeria. *Microorganisms*, 9(7): 1359, doi: 10.3390/microorganisms9071359.

Borjan D, Leitgeb M, Knez Ž, et al. 2020. Microbiological and antioxidant activity of phenolic compounds in olive leaf extract. *Molecules*, 25(24): 5946, doi: 10.3390/molecules25245946.

Brahim N, Karbout N, Dhaouadi L, et al. 2021. Global landscape of organic carbon and total nitrogen in the soils of oasis ecosystems in southern Tunisia. *Agronomy*, 11(10): 1903, doi: 10.3390/agronomy11101903.

Brahmi F, Mechri B, Dabbou S, et al. 2012. The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations. *Industrial Crops and Products*, 38: 146–152.

Condelli N, Caruso M C, Galgano F, et al. 2015. Prediction of the antioxidant activity of extra virgin olive oils produced in the Mediterranean area. *Food Chemistry*, 177: 233–239.

Connor D J. 2005. Adaptation of olive (*Olea europaea* L.) to water-limited environments. *Australian Journal of Agricultural Research*, 56(11): 1181–1189.

Connor D J, Fereres E. 2010. The physiology of adaptation and yield expression in olive. *Horticultural Reviews*, 31: 155–229.

Corona G, Deiana M, Incani A, et al. 2009. Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. *Molecular Nutrition and Food Research*, 53(7): 897–903.

Datt B. 1999. Remote sensing of water content in *Eucalyptus* leaves. *Australian Journal of Botany*, 47(6): 909–923.

de Bock M, Thorstensen E B, Derraik J G, et al. 2013. Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (*Olea europaea* L.) leaf extract. *Molecular Nutrition and Food Research*, 57(11): 2079–2085.

de Marino S, Festa C, Zollo F, et al. 2014. Antioxidant activity and chemical components as potential anticancer agents in the olive leaf (*Olea europaea* L. cv Leccino.) decoction. *Anti-Cancer Agents in Medicinal Chemistry*, 14(10): 1376–1385.

Dhia G, Benchaben H, Abdelkrim H. 2018. Foliar behavior of olive trees (*Olea europaea* L.) grafted and cut under the effect of salt stress. *Ukrainian Journal of Ecology*, 8(1): 578–584.

di Donna L, Mazzotti F, Naccarato A, et al. 2010. Secondary metabolites of *Olea europaea* leaves as markers for the discrimination of cultivars and cultivation zones by multivariate analysis. *Food Chemistry*, 121(2): 492–496.

Diaz-Espejo A, Buckley T N, Sperry J S, et al. 2012. Steps toward an improvement in process-based models of water use by fruit trees: A case study in olive. *Agricultural Water Management*, 114: 37–49.

Douzane M, Daas M S, Meribai A, et al. 2021. Physico-chemical and sensory evaluation of virgin olive oils from several Algerian olive-growing regions. *Oilseeds and Fats, Crops and Lipids*, 28: 55, doi: 10.1051/ocl/2021044.

Ennajeh M, Vadel A M, Cochard H, et al. 2010. Comparative impacts of water stress on the leaf anatomy of a drought-resistant and a drought-sensitive olive cultivar. *Journal of Horticultural Science and Biotechnology*, 85(4): 289–294.

Georgé S, Brat P, Alter P, et al. 2005. Rapid determination of polyphenols and vitamin C in plant-derived products. *Journal of Agricultural and Food Chemistry*, 53(5): 1370–1373.

Gharibi S, Tabatabaei B E S, Saeidi G, et al. 2019. The effect of drought stress on polyphenolic compounds and expression of flavonoid biosynthesis related genes in *Achillea pachycephala* Rech.f. *Phytochemistry*, 162: 90–98.

Gilani A H, Khan A U. 2010. Medicinal value of combination of cholinergic and calcium antagonist constituents in olives. In: Preedy V R, Watson R R. *Olives and Olive Oil in Health and Disease Prevention*. Amsterdam: Elsevier, 835–843.

Goldsmith C D, Vuong Q V, Sadeqzadeh E, et al. 2015. Phytochemical properties and anti-proliferative activity of *Olea europaea* L. leaf extracts against pancreatic cancer cells. *Molecules*, 20(7): 12992–13004.

Gomes S, Martins-Lopes P, Guedes-Pinto H. 2012. Olive tree genetic resources characterization through molecular markers. In: Caliskan M. *Genetic Diversity in Plants*. Croatia: InTech Publisher, 15–28.

Guerfel M, Baccouri O, Boujnah D, et al. 2009. Impacts of water stress on gas exchange, water relations, chlorophyll content and leaf structure in the two main Tunisian olive (*Olea europaea* L.) cultivars. *Scientia Horticulturae*, 119(3): 257–263.

Guinda Á, Castellano J M, Santos-Lozano J M, et al. 2015. Determination of major bioactive compounds from olive leaf. *LWT - Food Science and Technology*, 64(1): 431–438.

Hashim Y Z Y, Rowland I R, McGlynn H, et al. 2008. Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. *International Journal of Cancer*, 122(3): 495–500.

Hassen I, Casabianca H, Hosni K. 2015. Biological activities of the natural antioxidant oleuropein: Exceeding the expectation – A mini-review. *Journal of Functional Foods*, 18: 926–940.

Ilarioni L, Proietti P. 2014. Olive tree cultivars. In: Peri C. *The Extra-Virgin Olive Oil Handbook*. Oxford: Wiley, 5: 59–67.

IOC (International Olive Council). 1997. Methodology for the primary characterization of olive cultivars. In: RESGEN CT Project (67/97). European Union, IOC. Madrid, Spain.

Jones R J, Palmer B. 2000. In vitro digestion studies using $^{14}$C-labelled polyethylene glycol (PEG) 4000: comparison of six tanniniferous shrub legumes and the grass *Panicum maximum*. *Animal Feed Science and Technology*, 85(3–4): 215–221.

Karonen M, Oraviita M, Mueller-Harvey I, et al. 2015. Binding of an oligomeric ellagitannin series to bovine serum albumin (BSA): Analysis by isothermal titration calorimetry (ITC). *Journal of Agricultural and Food Chemistry*, 63(49): 10715–10722.

Kermanshah Z, Samadanifard H, Moghaddam O M, et al. 2020. Olive leaf and its various health-benefitting effects: A review study. *Pakistan Journal of Medical and Health Sciences*, 14(2): 1301–1312.

Lichtenthaler H K, Buschmann C. 2001. Extraction of photosynthetic tissues: Chlorophylls and carotenoids. In: Wrolstad R E, Acree T E, Decker E A. *Current Protocols in Food and Analytical Chemistry*. New York: Wiley, F.4.2.1-F.4.2.6.

Lins P G, Pugine S M P, Scatolini A M, et al. 2018. In vitro antioxidant activity of olive leaf extract (*Olea europaea* L.) and its protective effect on oxidative damage in human erythrocytes. *Heliyon*, 4(9): e00805, doi: 10.1016/j.heliyon.2018.e00805.

Maayan I, Shaya F, Ratner K, et al. 2008. Photosynthetic activity during olive (*Olea europaea*) leaf development correlates with plastid biogenesis and Rubisco levels. *Physiologia Plantarum*, 134(3): 547–558.

Malterre H. 1961. Rational nomenclature of the textures of soils and loose or calcareous rocks. *French Association for Soil Studies newsletter*. France: Special Issue, 27–40 (in French).

Markhali F S, Teixeira J A, Rocha C M. 2020. Olive tree leaves a source of valuable active compounds. *Processes*, 8(9): 1177, doi: 10.3390/pr8091177.

Marsh K J, Wallis I R, Kulheim C, et al. 2020. New approaches to tannin analysis of leaves can be used to explain in vitro biological activities associated with herbivore defence. *New Phytologist*, 225(1): 488–498.

Martín-García B, Verardo V, León L, et al. 2019. GC-QTOF-MS as valuable tool to evaluate the influence of cultivar and sample time on olive leaves triterpenic components. *Food Research International*, 115: 219–226.

Martín-García B, de Montijo-Prieto S, Jiménez-Valera M, et al. 2022. Comparative extraction of phenolic compounds from olive leaves using a sonotrode and an ultrasonic bath and the evaluation of both antioxidant and antimicrobial activity. *Antioxidants*, 11(3): 558, doi: 10.3390/antiox11030558.

Medina E, Romero C, García P, et al. 2019. Characterization of bioactive compounds in commercial olive leaf extracts, and olive leaves and their infusions. *Food and Function*, 10(8): 4716–4724.

Mehalaine S, Chenchouni H. 2020. Plants of the same place do not have the same metabolic pace: soil properties affect differently essential oil yields of plants growing wild in semiarid Mediterranean lands. *Arabian Journal of Geosciences*, 13: 1263, doi: 10.1007/s12517-020-06219-4.

Miliauskas G, Venskutonis P R, van Beek T A. 2004. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry*, 85(2): 231–237.

Mohamed M B, Gusami F, Ali S B, et al. 2018. The LC-MS/MS characterization of phenolic compounds in leaves allows classifying olive cultivars grown in South Tunisia. *Biochemical Systematics and Ecology*, 78: 84–90.

Moilanen J, Karonen M, Tähtinen P, et al. 2016. Biological activity of ellagitannins: Effects as anti-oxidants, pro-oxidants and metal chelators. *Phytochemistry*, 125: 65–72.

Muzzalupo I, Vendramin G G, Chiappetta A. 2014. Genetic biodiversity of Italian olives (*Olea europaea*) germplasm analyzed by SSR markers. *The Scientific World Journal*, 2014: 296590, doi: https://doi.org/10.1155/2014/296590.

Nelson D W, Sommers L E. 1983. Total carbon, organic carbon, and organic matter. In: Page A L, Miller R H, Keeney D R. *Methods of Soil Analysis Part 2, Chemical and Microbiological Properties*. Agronomy Monograph No. 9. Madison: American Society of Agronomy, 539–579.

Nelson R E. 1983. Carbonate and gypsum. In: Page A L, Miller R H, Keeney D R. *Methods of Soil Analysis Part 2, Chemical and Microbiological Properties*. Agronomy Monograph No. 9. Madison: American Society of Agronomy, 181–197.

Niyogi K K, Björkman O, Grossman A R. 1997. The roles of specific xanthophylls in photoprotection. *Proceedings of the National Academy of Sciences*, 94(25): 14162–14167.

Omar Z, Bouajila A, Brahim N, et al. 2017. Soil property and soil organic carbon pools and stocks of soil under oases in arid regions of Tunisia. *Environmental Earth Sciences*, 76: 415, doi: 10.1007/s12665-017-6745-z.

Ozkaya M T, Cakir E, Gokbayrak Z, et al. 2006. Morphological and molecular characterization of Derik Halhali olive (*Olea europaea* L.) accessions grown in Derik-Mardin province of Turkey. *Scientia Horticulturae*, 108(2): 205–209.

Parida A K, Dagaonkar V S, Phalak M S, et al. 2007. Alterations in photosynthetic pigments, protein and osmotic components in cotton genotypes subjected to short-term drought stress followed by recovery. *Plant Biotechnology Reports*, 1: 37–48.

Pereira A P, Ferreira I C F R, Marcelino F, et al. 2007. Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. Cv. Cobrançosa) leaves. *Molecules*, 12(5): 1153–1162.

Petridis A, Therios I, Samouris G. 2012. Genotypic variation of total phenol and oleuropein concentration and antioxidant activity of 11 Greek olive cultivars (*Olea europaea* L.). *HortScience*, 47(3): 339–342.

Pita P, Pardos J A. 2001. Growth, leaf morphology, water use and tissue water relations of *Eucalyptus globulus* clones in response to water deficit. *Tree Physiology*, 21(9): 599–607.

Rahmani R, Beaufort S, Villarreal-Soto S A, et al. 2019. Kombucha fermentation of African mustard (*Brassica tournefortii*) leaves: chemical composition and bioactivity. *Food Bioscience*, 30: 100414, doi: 10.1016/j.fbio.2019.100414.

Robinson G W. 1922. A new method for the mechanical analysis of soils and other dispersions. *The Journal of Agricultural Science*, 12(3): 306–321.

Ruzzolini J, Peppicelli S, Andreucci E, et al. 2018. Oleuropein, the main polyphenol of *Olea europaea* leaf extract, has an anti-cancer effect on human BRAF melanoma cells and potentiates the cytotoxicity of current chemotherapies. *Nutrients*, 10(12): 1950, doi: 10.3390/nu10121950.

Salah M B, Abdelmelek H, Abderraba M. 2012. Study of phenolic composition and biological activities assessment of olive leaves from different varieties grown in Tunisia. *Medicinal Chemistry*, 2(5): 107–111.

Sarker U, Oba S. 2018. Drought stress enhances nutritional and bioactive compounds, phenolic acids and antioxidant capacity of *Amaranthus* leafy vegetable. *BMC Plant Biology*, 18(1): 258, doi: 10.1186/s12870-018-1484-1.

Schofield P, Mbugua D M, Pell A N. 2001. Analysis of condensed tannins: A review. *Animal Feed Science and Technology*, 91(1–2): 21–40.

Shaheen M A, Hegazi A A, Hmmam I S A. 2011. Effect of water stress on vegetative characteristics and leaves chemical constituents of some transplants olive cultivars. *American-Eurasian Journal of Agricultural and Environmental Sciences*, 11(5): 663–670.

Singleton V L, Rossi J A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16: 144–158.

Talhaoui N, Taamalli A, Gomez-Caravaca A M, et al. 2015. Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits. *Food Research International*, 77: 92–108.

Tekaya M, El-Gharbi S, Mechri B, et al. 2016. Improving performance of olive trees by enhancement of key physiological parameters of olive leaves in response to foliar fertilization. *Acta Physiologiae Plantarum*, 38: 101, doi: 10.1007/s11738-016-2122-x.

Terzuoli E, Giachetti A, Ziche M, et al. 2016. Hydroxytyrosol, a product from olive oil, reduces colon cancer growth by enhancing epidermal growth factor receptor degradation. *Molecular Nutrition and Food Research*, 60(3): 519–529.

Tognetti R, Giovannelli A, Lavini A, et al. 2009. Assessing environmental controls over conductances through the soil-plant-atmosphere continuum in an experimental olive tree plantation of southern Italy. *Agricultural and Forest Meteorology*, 149(8): 1229–1243.

Vinha A F, Ferreres F, Silva B M, et al. 2005. Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): Influences of cultivar and geographical origin. *Food Chemistry*, 89(4): 561–568.

Walkley A, Black I A. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Science*, 37(1): 29–38.

Wang B, Qu J, Feng S, et al. 2019. Seasonal variations in the chemical composition of Liangshan olive leaves and their antioxidant and anticancer activities. *Foods*, 8(12): 657, doi: 10.3390/foods8120657.

Zaanouni N, Gharssallaoui M, Eloussaief M, et al. 2018. Heavy metals transfer in the olive tree and assessment of food contamination risk. *Environmental Science and Pollution Research*, 25: 18320–18331.

Zeitoun M A M, Mansour M M, Ezzat S, et al. 2017. Effect of pretreatment of olive leaves on phenolic content and antioxidant activity. *American Journal of Food Technology*, 12(2): 132–139.

Zhishen J, Mengcheng T, Jianming W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, 64(4): 555–559.

*Note: Figure translations are in progress. See original paper for figures.*