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Research Article

Climate change effects on phytochemical compounds and antioxidant activity of *Olea europaea* L.

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Abstract

To tolerate water shortage and high temperature, olive cv. 'Meski', the main variety of table olives in Tunisia, developed several biochemical changes. The hereby study focused on the adaptation of the olive tree to the climatic conditions, considering the evaluation of phenolic compounds, chlorophylls, carotenoids, saponin and steroid synthesis, as well as the evaluation of the antioxidant activity. The analyses were made upon fresh leaves collected from three coastal areas of Tunisia (North (sub-humid), Center (higher semi-arid) and South (lower arid)) and using different leaves' extracts. The results emphasized that Southern 'Meski' trees had leaves with more polyphenols, flavonoids, o-diphenols and tannins compared with the Northern ones. From the North to the South, 'Meski' leaves showed an increase of carotenoids and a decrease of chlorophyll *a* and *b* contents. The highest level of antioxidant compounds of Southern leaves could be contributed to reduce the oxidative stress of the olive tree. The spectrophotometric analysis of the antioxidant capacity of leaves collected from Central and Southern areas, based on DPPH and ABTS radical-scavenging activity, showed a higher value of antioxidant activity than the Northern ones, at different extract concentrations. Therefore, the increase of the analyzed bioactive compounds can be considered as a response of the tree to surround aggressions and to oppose the oxidative stress that results from the severity of climatic conditions, characteristic of the Southern area.

Keywords: bioactive compounds; biological properties; climatic conditions; leaves; olive tree

Introduction

Olive tree (*Olea europaea* L.) is one of the most important crops in the Mediterranean regions. Their cultivation in Tunisia is of great agronomic and socio-economic importance. The Tunisian olive forest covers 1.8 million ha and has around 80 million olive trees distributed throughout the country (DGPA, 2015). More than one third of the country's agricultural land is devoted to olive growing. The cultivation of the olive tree is spread from the North to the South, in varying bioclimatic conditions, with a great varietal diversity. Tunisia thus occupies the fourth position in the world in number of trees and the second largest in regard with the olive trees' cultivated area. At the national level, it contributes to the achievement of national objectives of food

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security, preserving natural resources and limiting rural exodus. Internationally, it gives Tunisia the rank of the fourth largest producer and third largest exporter of olive oil.

Several studies and experimental research have established that traditional Mediterranean diet is a source of good health (Keys, 1995), whereas the positive effect is linked to a main component of this diet, which is the olive and its derivatives. The fruit contain an important source of phenolic and antioxidant compounds. The derivate products have natural antioxidant properties, which intervene in the prevention and treatment of cancer, inflammatory and cardiovascular diseases. They are also used as additives for the food, pharmaceutical and cosmetic industries (Aouidi *et al*., 2007). The interest in phenolic compounds is extended to all olive products that can be consumed as foods, medicines, or that are generated as by-products of the olive industry such as leaves, wood and oils.

Olive trees are well adapted to abiotic stress (Zhang *et al*., 2011). The severity of climate caused biochemical changes in olive trees, such as the presence of secondary metabolites. These compounds played an important role in the plant response to environmental stress, having an important bioactivity against oxidative stress (Abaza *et al*., 2015). Recently, scientific research is increasingly interested in the leaves of olive tree, known for their richness in bioactive compounds such as vitamins, flavonoids and polyphenols. According to Aouidi *et al*. (2007), the highest percentage of total phenols is reserved for leaves, compared to other olive trees' organs, with a percentage of 35%. Olive leaves have interesting antimicrobial and antioxidant activities and can be used as a natural and functional ingredient in food technology (Taamalli *et al*., 2012).

Moreover, several abiotic and biotic factors may be involved in the quantitative and qualitative variation of the various biochemical compounds of the olive tree. The climatic conditions exert the most significant effects, generated by a gradual restriction of rainfall and a continuous rise in temperature (Brahmi *et al*., 2012). Nevertheless, the antioxidant compounds, especially in the olive tree, are very sensitive to changes of climatic factors, which are increasing from day to day, resulting in significant global warming. Environmental stresses, especially the severity of climatic factors, significantly modify the metabolism of polyphenols, as well as the concentration of secoiridoids in olive trees (Petridis *et al*., 2012). The olive tree responds with the regulation of antioxidants synthesis to oppose stress.

The aim of the current work was to determine the total phenol, flavonoid, o-diphenols, tanin, chlorophyll, carotenoids, phytosterols and saponin contents in methanol extracts from 'Meski' olive cultivar leaves, collected within different climatic conditions. In addition, the antioxidative properties of the same cultivar in different bioclimatic levels were evaluated by DPPH and ABTS $^{\bullet+}$radical-scavenging assays, in an attempt to establish the response and the adaptation of the olive cultivar against the severity of climate.

Materials and Methods

Plant material

Fresh leaves of 'Meski' cultivar were collected from three coastal zones of Tunisia: in Morneg (36° 40′51″North; 10° 17 25″Est), Chott Mariem (35° 56′08″N; 10° 33′26″E) and Zarzis (33° 30′N; 11°07′E) (Figure 1). Sampling was realized from different branches from twelve olive trees, about thirty years old. These samples were selected according to four orientations of the trees (North, East, South and West). 'Meski' cultivar has been grown on extensive, rainfall farming, in orchards characterized with densities up to 100 trees/ha. The relative humidity, the temperature and the precipitation of the studied areas were estimated from a standard meteorological station adjacent to the experimental field (Figure 2).

Preparation of leaf extracts

After harvest, the leaves were washed well with distilled water and air-dried at room temperature and in the shade. After grinding, the vegetable powder was stored away from light and humidity until extraction. The

5 g of sample dry weight was soaked in 50 mL of distilled water and left at room temperature, with stirring for 2 days. Thus, it is the hydraulic maceration that has been adopted to prepare the aqueous extract.

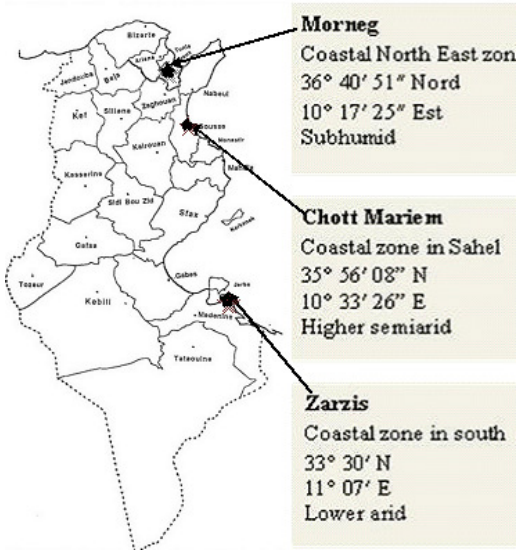

Figure 1. Description of three Tunisian growing areas of olive cv. 'Meski' Plant collected zones are shown in asterisk

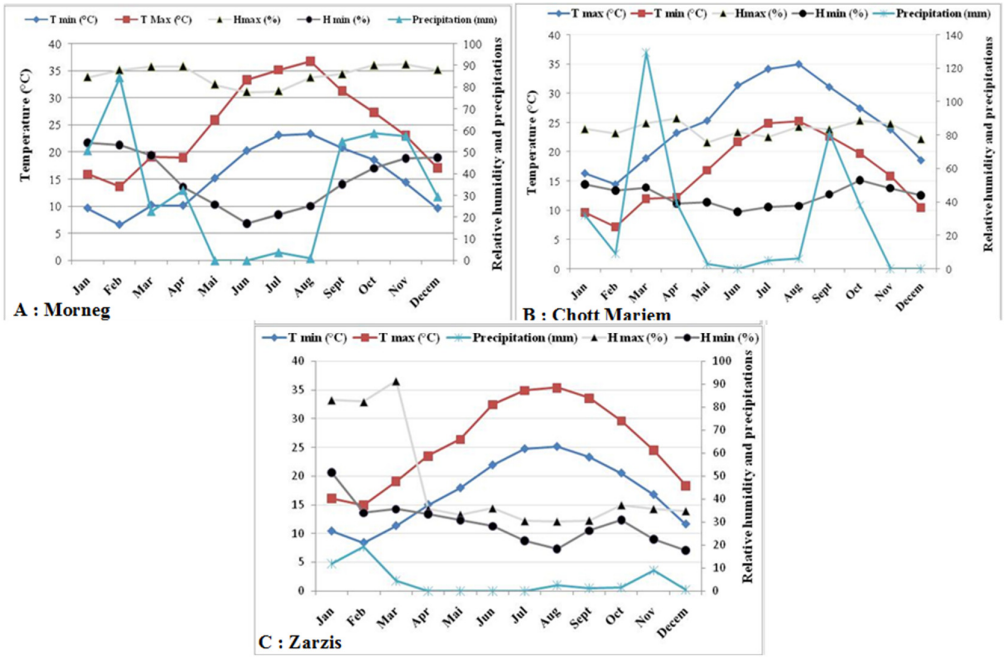

Figure 2. Monthly average of temperature, relative humidity and precipitations of three Tunisian studied areas during the culture 2012 of Olive tree Meski (Tmax: Maximal Temperature; Tmin: Minimal Temperature and HR: Relative Humidity)

In order to carry out analyzes of the phenolic compounds by colorimetric and chromatographic methods, the methanolic extract was prepared by mixing the dried aqueous extracts in methanol, and stirring for 24 hours. Then, a series of filtrations were carried out before drying by a rotary evaporator.

Determination of secondary metabolites content

Determination of total phenols

Total phenolic content of the methanolic extracts was spectrometrically analyzed at 725 nm using the modified method designed by Ryan *et al*. (1999). The concentration of total polyphenols was estimated with Folin-Ciocalteau reagent. To 100 µL of each sample extract suitably diluted, 750 µL of Folin-Ciocalteu reagent and 100 µL of di-ionized water were added. The mixture was shaken, added with 750 µL of Na_2CO_3 (6%, w/v) and adjusted with distilled water to a final volume of 3 mL. After incubation in darkness for 90 min at 23 °C, the absorbance versus prepared blank was recorded at 725 nm. The total phenolic compound (TPC) was expressed as milligram cathechin equivalents per gram of dry weight (mg CE.g^{-1} DW) cathechin using a calibration curve with (10-50 µg·mL^{-1}).

Determination of total flavonoids

Total flavonoids of the dry 'Meski' leaves extracts was spectrophotometrically determined by the aluminum chloride method, using quercetin as standard, as performed by the method of Dehpeur *et al*. (2009). One ml of properly diluted leaves' extract was mixed with 4 mL of distilled water. Then, 0.3 mL of NaNO2 (5%, w/v) were added. After five minutes, 0.3 mL of (10%, w/v) AlCl3 and 2 mL of NaOH (1 M) were added. Finally, the volume was immediately made up to 10 ml, by the addition of 2.4 ml of distilled water. After 30 min incubation at darkness in room temperature, the absorbance of samples was measured at 430 nm. The results were expressed as mg quercetin equivalents (QE)/g dry weight. The values were calculated using the following equation: $Y = 0.035x + 0.288$ with $R^2 = 0.995$. Samples were analyzed in triplicates.

Determination of condensed tannins

The condensed tannins are determined using the acidic vanillin method described by Ba *et al*. (2010). The method is based on the ability of vanillin to react with units of condensed tannins in the presence of an acid- hydrogen chloride, to produce a slightly coloured complex, measured at 510 nm. The vanillin reagent was prepared by mixing equal volume: 8% (v/v) HCI, 37% (v/v) methanol and 4% vanillin in methanol (w/v). 200 mL of each extract were added to 1,000 mL of vanillin reagent; the mixture was stirred and then incubated in the dark at 30 °C for 20 min. The absorbance was measured at 510 nm by a UV spectrophotometer (JENWAY 6400) against a blank consisting of a mixture of methanol (37%) and HCl (8%) at equal volume. The results are expressed in mg catechin equivalents/g of dry weight with reference to the catechin calibration curve as follows:

$Y = 0.0053x + 0.0075$ with $R^2 = 0.9993$.

Determination of o-diphenols

The total content of the plant extract in diphenol was estimated by the method of Blekas et *al*. (2002), thus the determination of the extract with a solution of sodium molybdate (catalyst) in an acid and in the presence of phosphate buffer. After incubating at room temperature, the solution was analysed at 350 nm in UV-visible spectrophotometer. Caffeic acid was used to prepare a standard range in the concentration range of 0 to 100 mg. L^{-1}.

Determination of pigment content

The methanolic extract of each sample was determined by UV spectrophotometer and the absorbances were read at 400-700 nm. It was recorded that chlorophyll *a* showed the maximum absorbance at 662 nm, chlorophyll *b* at 646 nm and total carotenoids at 470 nm, whereas the amount of these pigments were calculated according to Lichtenthaler and Buschmann (2001):

$Ca = 15{,}65\ A_{666} - 7{,}340\ A_{653}$

$Cb = 27,05\ A_{653} - 11,21\ A_{666}$

$C_{x+c} = 1000\ A_{470} - 2,860\ Ca - 129,2\ Cb/245$

Chlorophyll *a*, *b* and carotenoids contents were expresses in $mg.g^{-1}$ of weight extract.

Determination of saponin

The total saponin content in methanol extract was assayed according to the method described by Baccou *et al*. (1977). The standard solutions of dioscin were prepared, at the concentration 10.35 mg in 50 mL of methanol, to produce the reference dioscin. For the calibration curve, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 0.9 mL standard solutions were individually added into one tube. Then, in the test tube, 200 µl of 5% vanillin and 800 µL of perchloric acid were added. Afterward, this tube was placed in a water bath maintained at 60 °C for 20 min and then allowed to cool in 0 °C water bath, after which 5 ml glacial acetic acid were added. The absorbance of the solution was measured at 457 nm by using a UV-visible spectrophotometer. An amount of 200 µL of sample extract, at the concentration of 50 $mg.mL^{-1}$, was used. The results were expressed in mg Dioscine equivalent/g of extract.

Determination of steroids

The determination of phytosterols was based on the direct contact of the plant extract and the Liebermann-Burchard (LB) reagent (Araújoa *et al*., 2013). The latter is a mixture of three strong acids: glacial acetic acid, sulfuric acid and cold anhydride acetic. Theβ-Sitosterol was used as a standard, with a concentration of 0.02 to 0.1 $mg.mL^{-1}$. The mixture contains 200 of the extract and 5 µL of LB, was read in UV-visible spectrophotometer at 625 nm.

Determination of antioxidant activity

The determination of antioxidant capacity of the methanolic extracts was tested by DPPH radical scavenging and ABTS radical cation assays as follows.

DPPH radical-scavenging assay

The antioxidant activity of 'Meski' leaves methanolic extracts was measured according to the method of Choi *et al*. (2002). The method is based on the radical scavenging ability against DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate). Samples of methanol extracts (0.5 mL) with different concentrations (1,000; 250; 62.5; 3.906 and 0.976 µg/mL) were added to 0.5 mL of a DPPH methanolic solution (60 µM). After stirring vigorously, the mixture was left standing in the dark at room temperature for 30 min. The absorbance was read at 520 nm using ethanol as blank. 0.5 mL of DPPH solution mixed with 0.5 mL of ethanol was used as control. All the analyses were made in triplicate and total antioxidant activity was expressed as the percentage inhibition of the DPPH radicals scavenging rate and was determined as:

$$\text{DPPH radical scavenging activity (\%)} = [1- (A_{sample}/A_{control})] \times 100$$

Where $A_{control}$ is the absorbance of the control after 30 mn (containing all reagents except the extract compound), A_{sample} is the absorbance of the tested sample after 30 mn.

The actual decrease in absorbance induced by the tested sample (change of colour from deep-violet to light yellow) was compared to that of the positive control trolox. TEAC (Trolox equivalent antioxidant capacity) was determined as the amount of Trolox equivalent to the amount of test substance that resulted in 50% scavenging of DPPH radicals.

The IC50 values represent the concentration ($mg.mL^{-1}$) of sample which is required to scavenge 50% of DPPH free radicals.

Experiments were carried out in triplicate and the mean value was recorded.

ABTS •+radical assay

The evaluation of antioxidant activity for tested extracts was based on the $ABTS^{\bullet+}$ free radical decolorization assay according to the method of Yu *et al*. (2002). The $ABTS^{\bullet+}$ is widely used to assess the total amount of radicals that can be scavenged by an antioxidant capacity. Radical cation was prepared by oxidizing 100µL of ABTS commercial solution with 0.1 mg of $K_2S_2O_8$. The mixture was diluted in 100 mL of di-ionised water and incubated in the dark at room temperature for 24 h. Samples were diluted in ethanol at different concentration (1,000; 250; 62.5; 3.906 and 0.976 $\mu g.mL^{-1}$). The volume of 10 µL of ethanolic fractions of 'Meski' leaves were added, at different concentrations, to 990 µL $ABTS^{\bullet+}$solution and the absorbance was noted after 5, 10, 15, 20 and 30 min of initial mixing, at room temperature.

The absorbance of all samples was compared to that of the calibrated Trolox (TE) standard. The free radical-scavenging activity of each extract was measured in terms of Trolox equivalent antioxidant capacity (TEAC) as described by Re *et al*. (1999), measured after 30 min of the beginning of the reaction and the results were expressed as percent of inhibition. All experiments were carried out in triplicate. In fact, TEAC analysis is widely used to estimate the number of radicals inhibited by an antiradical (Arts *et al*., 2004).

Results and Discussion

Total phenols and o-diphenols contents

The determination of total phenols content (TPC) in leaves' methanolic extracts (Figure 3) revealed a significant difference between studies areas. The means of TPC in olive leaf extracts in terms of mg (cathechin equivalent) $CE.g^{-1}$ Dry Weight (DW) were 1.45 ± 0.0, 2.73 ± 0.01 and 2.97 ± 0.03 mg $CE.g^{-1}$ DW for Northern, Center and Southern 'Meski' olive leaves respectively. In fact, 'Meski' leaves growing in the South and in the Center seemed to be the richest in phenolic compounds, contrarily to the Northern ones, which presented the lowest levels. These results were in accordance with the report of Brahmi *et al*. (2012), who showed, for the methanolic extract of Nebjmel, a seasonal change in the total phenol content between 3.51 and 2.50 $mg.g^{-1}$ DW from leaves collected in October and February respectively.

The analysis of o-diphenols content in leaves extracts of cv. 'Meski' showed a significant difference between studied aeres (Figure 3B). The concentration of o-diphenols in 'Meski' leaves changed depending on the origin of samples and collected area. In fact, the highest level of this compound was found in extracts obtained from leaves collected in the Center of Tunisia (2.09 ± 0.40 $mg.g^{-1}$ DW). However, Northern leaves showed the lowest contents such as 1.26 ± 0.12 $mg.g^{-1}$ DW. Phenolic compounds, such as phenols and o-diphenols in plants constitute a major class of secondary plant metabolites with bioactive potential attributed to antioxidant activity (Brahmi *et al*., 2012). Other studies report high polyphenolic content in extracts which was associated with antioxidant activity (Turkoglu *et al*., 2007). Thus, to protect against oxidative stress, procured by harsh weather conditions, 'Meski' olive tree improved phenolic content in their leaves.

Flavonoid contents

Flavonoids are one of the most important natural phenolic compounds (Shimoi *et al*., 1996). Total flavonoid contents of different extracts from olive leaves (Figure 4.) showed a significant difference between the studied areas. Therefore, these levels varied considerably, from 0.023 ± 0.00 to 0.673 ± 0.00 $mg.g^{-1}$ DW respectively for the Northern, central and Southern 'Meski' leaves. The obtained results agree with Brahmi *et al*. (2012), who reported that the total flavonoids content in leaves extracts of Nebjmel varied with the season and the solvent extract. Also, Zeitoun *et al*. (2017) showed that flavonoids content of olive leaves ranged from 0.057 ± 0.006 to 0.125 ±0.003 $mg.g^{-1}$. The leaves extracts obtained from Southern area registered the highest level of total flavonoids. In fact, leaves in Southern area were twenty time richer in flavonoids than the

Northern ones. The increase of this antioxidant could be attributed to the severity of climate. In general, drought increased concentrations of antioxidants and photo protective pigments (Wujeska *et al.*, 2013).

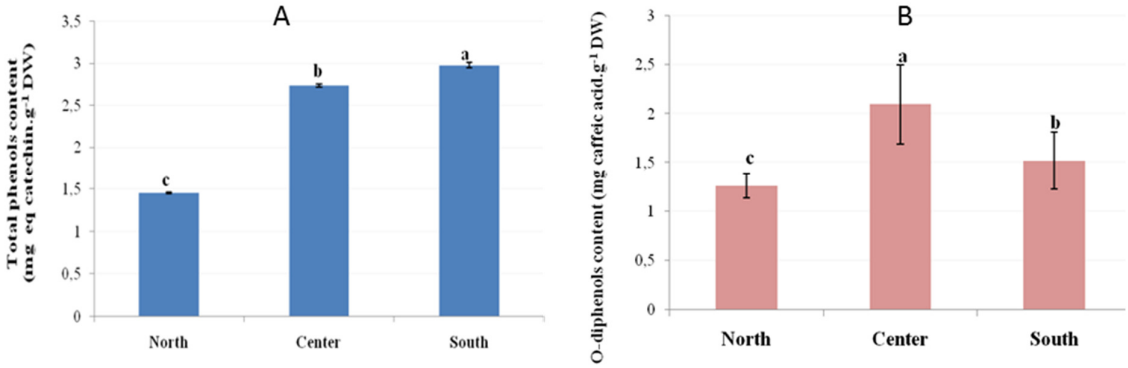

Figure 3. Total phenols (A) and o-diphenols contents (B) of extracts of olive leaves harvested at flowering. Results are expressed as cathechin and caffeic acid equivalents respectively for total phenols and o-diphenols. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of $p < 0.05$

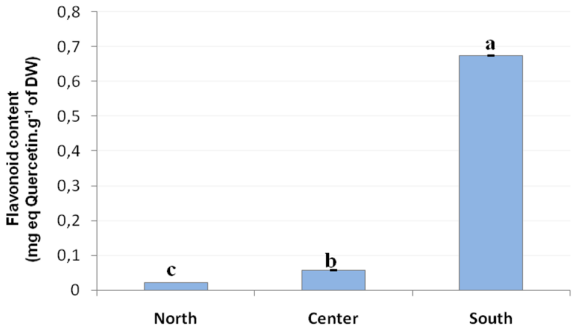

Figure 4. Total flavonoid contents of olive leaves harvested at flowering. Means with different letters were significantly different at the level of $p < 0.05$. Results are expressed as quercetin equivalents for total flavonoids in Center and Southern collected leaves, demonstrating their important role in adaptation to severe climatic conditions

Generally, drought increased the concentration of antioxidants (Wujeska *et al.*, 2013). Some flavonoids provide stress protection, as scavengers of free radicals such as reactive oxygen species (ROS) (Falcone Ferreyra *et al.*, 2012). These compounds could be considered as a marker of draught stress and adaptation.

Condensed tannins content

The condensed tannin values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of $p < 0.05$.

Condensed tannins, oligomeric and polymeric proanthocyanidins are natural sources of organic compounds, widely used in medicinal and industrial application (Morales *et al.*, 2017). According to the results obtained (Figure 5), leaves' extract showed a significant difference between the three areas. The extract of leaves collected from the South and the Center presented high condensed tannin content. In fact, the Southern (0.84 ± 0.00 mg EQ.g^{-1} DW) and the Center leaves (0.74 ± 1.36 mg EQ.g^{-1} DW) registered the upmost level in condensed tannins, but the Northern ones (0.24 ± 0.00 mg EQ.g^{-1} DW) showed the lowest amount. Condensed tannins increased with the severity of climate, draught and high temperature. However, under favorable climates, in Northern area, the tannin concentrations in leaves tissues formed were lower. These

results are in agreement with those of Top *et al*. (2017) who proved that the climate stress in *Quercus ruba* responded by producing a greater quantity of tannins. These phenolic compounds could be attributed to protect plants against environmental stresses (Agrawel *et al*., 2005; Top *et al*., 2017).

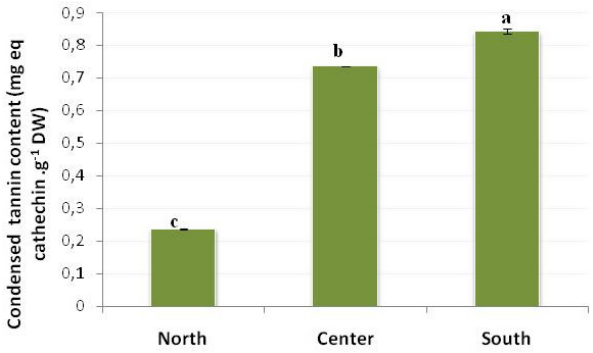

Figure 5. Condensed tannins contents of olive leaves extract collected from three different bioclimatic ranges of Tunisia. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of $p < 0.05$

Phytosterols content

Sterols are isoprenoid-derived compounds, essential to the growth and development of eukaryotic organisms. Sterols are lipophilic membrane components essential for diverse cellular functions, such as cell division, cell elongation, cell polarity and cellulose accumulation (Boutté and Grebe, 2009). Results showed a significant difference between different areas studied hereby. In fact, according to Figure 6, the highest content of phytosterol content was present in Southern leaves (2. 94 mg eq sitosterol. g^{-1} DW), followed by Center leaves (0.79 mg eq sitosterol.g^{-1} DW), whereas the Northern ones registered the lowest content (0.22 mg eq sitosterol.g^{-1} DW). The increase of phytosterols with the severity of climate could be a response of olive tree to support draught stress and indicated the role of this compound in providing tolerance to stress (Kolo, 2016). The result was in agreement with those of Vrieta *et al*. (2013) and Ferrer *et al*. (2017) who reported that these sterols are involved in plant growth and development, as well as in plant responses to biotic and abiotic stresses. There are increasing evidences supporting the involvement of conjugated sterols in plant stress responses, whereas sterols play a prominent role in plant stress tolerance (Vrieta *et al*., 2013; Ferrer *et al*., 2017). The study of Kolo (2016) showed that the inhibition of phytosterols synthesis increased the reactive oxigen species content in maize leaves.

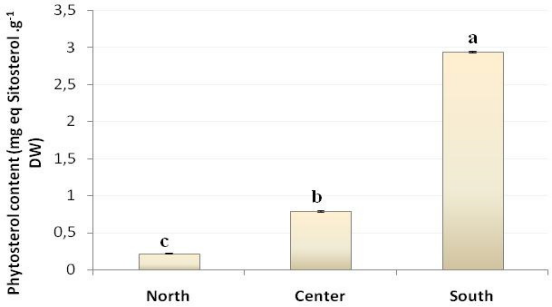

Figure 6. Phytosterol contents of 'Meski' leaves extract collected from different areas. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different (Duncan test, $p < 0.05$)

Saponin content

Saponins are natural phyto-chemicals and may be found in a wide variety of plants. Saponins are active ingredients in plants, consisting of a hydrophobic aglycone structure with hydrophilic sugar residues (Böttcher and Drusch, 2017). These properties are responsible for the interaction between saponins and cell membranes, attributes such as their anti-fungal activities (Madland, 2013), anti-oxidant, anti-virus, anti-inflammation and anti-cancer activities (Lv *et al*., 2013; Yu *et al*., 2014). These compounds have a promising application in medicine, agriculture, industry and environmental protection. The results obtained showed the effect of the region on saponin production in 'Meski' leaves and are presented in Figure 7. A significant difference between Northern, Central and Southern 'Meski' leaves saponin content was noted. Saponin content increased with the severity of climate. However, leaves collected from Southern areas (high temperature and draught) showed the highest saponin level (0.87 ± 0.00 mg eq diocin.g^{-1} DW), followed by the Center (0.678 ± 0.00 mg eq diocin.g^{-1} DW and the North (0.42 ± 0.00 mg eq diocin.g^{-1} DW). These results agreed with those of Odjegba and Alokolaro (2013) who reported that drought and salinity treatments enhanced saponin production in *A. wilkesiana* plants. Also, El-Sayed *et al*. (2008) reported that saponin content in *Trubulus* increased under water stress. The increase could be related to the protective role against oxidative stress (Lin *et al*., 2009).

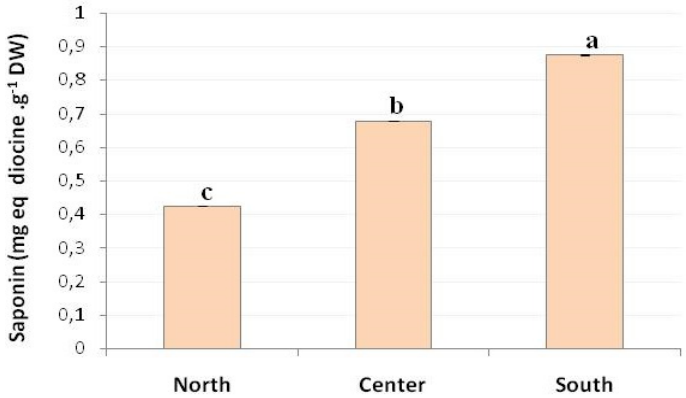

Figure 7. Saponin contents in leaves extract of 'Meski' collected from different areas. Values are expressed in mg equivalent diocine per gram of the plant extract, as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of $p < 0.05$

Pigment content

The analysis of pigments' content in the leaves showed significant differences between studied areas. The concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids were influenced by climatic conditions (Figure 8). The highest chlorophyll levels were registered in Northern 'Meski' leaves, but the lowest was occurred in Southern ones (total chlorophyll content was 225.80 ± 22.54, 149.10 ± 6.32 and 77.67 ± 0.59 mg.g^{-1} DW respectively for Northern, Center and Southern leaves). Chlorophyll *a*, *b* and total chlorophyll levels decreased with the severity of climate, such as the increase of temperature and draught. The results were in accordance with those of Hanci and Cebeci (2014) and Arji and Arzani (2008) who proved that under drought stress conditions, the amount of chlorophyll *a* and *b* in olive cultivars decreased significantly. Indeed, drought is one of the factors affecting photosynthesis and chlorophyll content (Khaleghi *et al*., 2012).

Carotenoids content in leaves extract increased with the severity of climate from the North to the South. In fact, the amount of carotenoids in Southern leaves extracts (122.80 ± 0.02 mg.g^{-1} DW) was higher than that in the Northern ones (4.94 ± 0.65 mg.g^{-1} DW). The significant increase in content of carotenoids in the studied cultivar under severe climate suggested that Southern olive leaves provided stronger photo protective system against oxidative stress caused by drought stress (Hanci and Cebeci, 2014). Also, Wujeska *et al*. (2013) attributed the increase of the concentration of antioxidants with progressing stress, as a strengthening of defense systems and therefore an indication of acclimation to stress. Some researchers reported that carotenoids

had an antioxidant property against oxidative stress. Therefore, maintenance of higher levels of the antioxidants can be a good strategy of plants to counter the negative effects of reactive oxygen species (Sharma and Dubey, 2005) by preventing chlorophyll-photosensitized formation of $^1O2$ by intercepting the chlorophyll triplet states (Demmig-Adams and Adams, 1996).

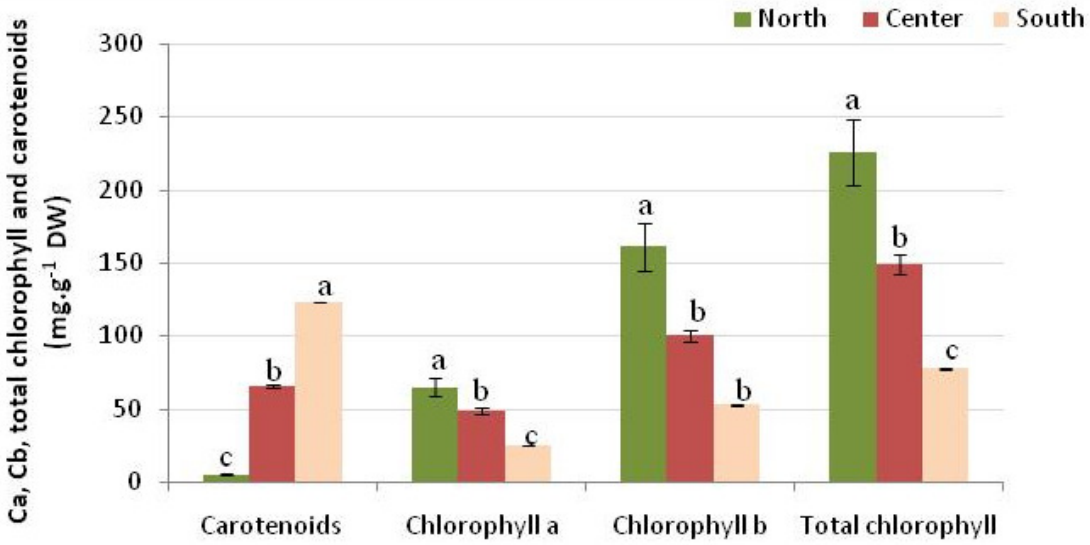

Figure 8. Total pigment content of the olive leaves sampled from cultivar 'Meski' harvested at flowering from different areas. Mean composition of sampled leaf tissue from three replications of three individual samples (three plants each) ± standard deviation

Antioxidants capacity of olive leaves

The capacity of olive leaves extracts, collected from different areas, to scavenging free radicals have been evaluated applying DPPH and $ABTS^{\bullet+}$ radicals.

Radical-scavenging activities on DPPH

The DPPH is a stable free radical. It accepts an electron or hydrogen radical to become a stable molecule and is reduced in the presence of an antioxidant (Choi, 2002). The evaluation of the antioxidants activities of 'Meski' leaves from Northern, Central and Southern areas were expressed in inhibition percentage and the results are established in Table 1.

The antioxidant in all investigated samples seemed to be proportional to the concentration of the extract. The antioxidant activity of 'Meski' leaves is very important, reaching up to 95.26% for the central areas at a concentration of 1,000 $\mu g.mL^{-1}$ and 76.58% for the Southern ones. Indeed, over than 62.5 $\mu g.mL^{-1}$ concentration, methanolic extracts of the Center area showed the highest antioxidant activity up to 83% against DPPH radicals. Meanwhile, at low concentration (0.976 $\mu g.mL^{-1}$) Southern leaves extract showed the most antioxidant activity against DPPH radicals (59.32 ± 0.68%). Indeed, above 62.5 $\mu g.mL^{-1}$ concentrations, the inhibition percentage of tested extracts was lesser than that of Trolox, but for the lower ones (15.62; 3.976 and 0.976 $g.mL^{-1}$), the inhibition of these extracts exceeds it, thus showing a better efficiency of these extracts to scavenging free radicals at low levels.

Table 1. Free radical-scavenging capacities of olive leaves extracts, Trolox and IC50 measured in DPPH assay

Concentrations (µg.mL^{-1})	1,000	250	62.5	15.625	3.906	0.976	IC50 (µg.mL^{-1})
Inhibition percentage (%)							
North	66.04 ± 0.44	73.18 ± 4.51	52.22 ± 2.75	39.06 ± 1.40	40.1 ± 3.90	40.88 ±7.20	50.7 ± 0.01
Center	95.27 ± 0.00	92.77 ± 1.12	83.77 ± 14.33	51.76 ± 1.62	47.15 ± 9.03	43.75 ±3.64	12.3± 0.04
South	76.59 ± 4.84	84.35 ± 6.45	79.99 ± 2.69	68.04 ± 1.13	58.61 ± 2.70	59.32 ±0.68	0.6± 0.02
Trolox	99.37± 0.01	99.37± 0.50	99.71± 1.10	32.05± 0.70	9.98± 0.91	3.56± 0.35	24.5± 0.01

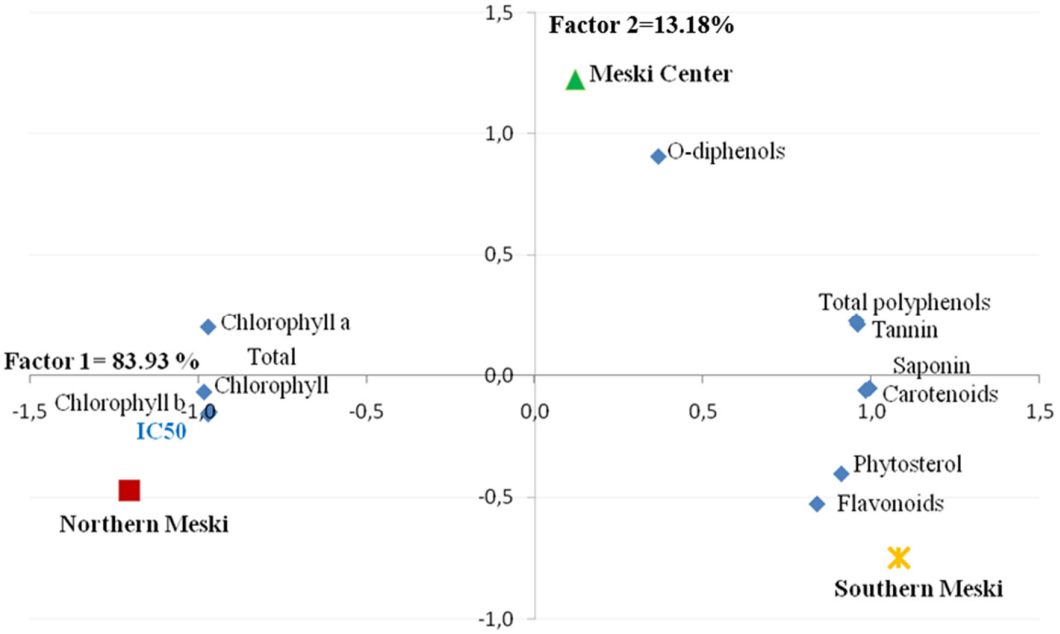

Figure 9. Principal component analysis of the main groups of phenolic compounds, pigment content, saponin, phytosterol in 'Meski' leaves from different areas (North, Center and South of Tunisia). Factors 1 and 2 explain 97.11 % of the data variation

In the three studied areas leaves extracts have shown important scavenging activities on DPPH radicals. Furthermore, the Southern and the Center leaves extract registered higher antioxidant activities than the Northern ones (IC50 = 50.7 µg.mL^{-1}). The lowest IC50 was represented by Southern leaf extract, 0.6 ± 0.02 µg.mL^{-1} suggesting a high antioxidant capacity of these leaves to neutralize free radicals. The results were lower than those of Zeitoun *et al.* (2017) and Thalhaoui *et al.* (2016) who fined in dried leaves IC50 values of 427.00 ± 23.5 µg.mL^{-1} and 129.9 ± 23.5 µg.mL^{-1} respectively. Furthermore, the hereby obtained results were in accordance with those of Boubakri *et al.* (2017), who explained that the IC50 were 44.83 ± 0.35 µg.mL^{-1} and 58.08 ± 3.5 µg.mL^{-1} of Swihli and Rihane extracts respectively.

According to the results presented in Table 1, Southern (IC50 = 0.6 ± 0.02 µg.mL^{-1}) and Center (IC50 = 12.3 ± 0.04 µg.mL^{-1}) leaves extracts were characterized by a higher free radicals scavenging property in comparison to that of synthetic antioxidant Trolox (IC50 = 24.5 µg. mL^{-1}). According to Zeitoun *et al.* (2017) antioxidant molecules such as phenolic compounds, flavonoids, tannins, carotenoids and phytosterols, which play part in the antioxidant system as non-enzymatic components, reduce and decolorize DPPH due to their hydrogen donating ability.

Therefore, maintenance of the higher levels of the antioxidants can be a good strategy by the plants to counter the negative effects of ROS generated by draught and high temperature (Sharma and Dubey, 2005).

$ABTS^{\bullet+}$ scavenging assay

The evaluation of ABTS radical cation scavenging capacity of the tested extracts were expressed as percentages of free radical inhibitions as function of time (Table 2).

The potential antioxidant of the extract of different leaves showed consistent variability among different studied areas. Also, the samples depended strongly with the duration of the reaction and the concentration of the extract.

Table 2. $ABTS^{\bullet+}$ cation radical scavenging capacity (%) of methanolic extracts of cv. 'Meski' leaves collected from the North, the Center and the South of Tunisia, as a function of time and concentrations

Origins	Incubation time	µg.mL^{-1}	5 min	10 min	15 min	20 min	30 min
Leaves	North	1,000	22.66±0.04	19.37±0.02	25.45±0.03	23.96±0.05	26.31±0.04
		250	22.21±0.01	11.04±0.04	12.39±0.03	12.03±0.01	14.82±0.03
		62.5	5.11±0.04	5.50±0.01	6.17±0.01	6.89±0.02	10.45±0.04
		15.62	4.34±0.04	4.28±0.01	6.53±0.01	4.59±0.02	10.95±0.02
		3.90	2.30±0.02	6.49±0.01	5.72±0.02	7.16±0.01	8.56±0.02
		0.97	3.69±0.02	4.64±0.01	4.37±0.08	7.79±0.02	7.84±0.01
	Center	1000	35.24±0.05	43.55±0.07	43.50±0.08	44.91±0.03	51.50±0.08
		250	20.12±0.1	22.03±0.01	22.12±0.09	22.30±0.04	29.39±0.06
		62.5	19.62±0.01	17.85±0.08	11.85±0.03	10.45±0.04	26.17±0.12
		15.62	18.94±0.14	18.03±0.13	19.63±0.07	19.99±0.06	25.58±0.02
		3.90	18.48±0.04	21.07±0.05	22.52±0.07	23.52±0.03	24.61±0.06
		0.97	18.43±0.14	19.39±0.04	19.26±0.02	20.99±0.02	24.48±0.02
	South	1000	30.78±0.01	30.29±0.02	32.79±0.08	38.70±0.04	43.82±0.01
		250	14.67±0.02	17.03±0.01	19.17±0.04	21.93±0.03	29.79±0.08
		62.5	11.04±0.02	10.49±0.01	13.44±0.04	20.44±0.08	25.80±0.02
		15.62	7.90±0.01	10.27±0.06	13.26±0.02	17.44±0.02	21.44±0.07
		3.9	7.58±0.02	8.08±0.01	11.12±0.01	16.71±0.1	21.04±0.01
		0.97	7.35±0.02	9.76±0.02	10.12±0.07	11.26±0.04	20.67±0.01

The antioxidant activity increased as function of time and the extract concentrations. In fact, at 1,000 µg.mL^{-1} after 30 min of contact with $ABTS^{\bullet+}$, 'Meski' leaves had a moderate antioxidant activity of about 51.50 ± 0.08, 43.82 ± 0.01 and 26.31 ± 0.04% for the Center, the Southern and the Northern leaves respectively. Indeed, at the same concentration and after 5 min of application only 22.66 ± 0.04, 35.24 ± 0.05 and 30.78 ± 0.01% of radicals were scavenged from Northern, Center and Southern 'Meski' leaves respectively (Table 2).

At the same incubation time (30 min), the activity depended to the concentration of the extracts. In fact, the antioxidant activity seemed to increase according to the concentration, especially for the central areas where there was a significant increase, around 68%.

For the lower concentrations, less than 250 µg.mL^{-1} for all tested areas, leaves extracts exhibited a feeble scavenging efficiency toward ABTS cation radicals, from 2.30 ± 0.02% to 29.79 ± 0.08% for Northern and Southern leaves respectively. These results were in agreement with those of Brahmi *et al*. (2014) which indicated an important impact of the region on the antioxidant potential of leaves, stems and fruits of 'Chemchali' and 'Chetoui', while they suggested that olive leaves cultivated in the North of Tunisia contained higher antioxidant activity than the leaves from the South.

The TEAC is a measurement of the effective antioxidant activity of the fraction, whereas a higher TEAC would imply greater antioxidant activity of the sample. It was observed that methanolic fraction of Southern leaves had the highest TEAC of 0.94 mM, when applied at 1,000 µg.mL^{-1} (Table 3). However, the TEAC values

of the other fractions, at this concentration, ranged from 0.12 to 0.74 mM. After 30 min of the initiation reaction, the TEAC of all extracts was lower than 1 mM (Table 3). So, the antioxidant activity of extracts of this variety is therefore lower than that of Trolox.

Table 3. Radical cation scavenging activity of leaves extracts expressed as Trolox equivalent after 30 min of incubation

Concentrations ($\mu g.mL^{-1}$)						
TEAC	1,000	250	62.5	15.62	3.9	0.97
North	0.74	0.73	0.39	0.53	0.28	0.27
Center	0.64	0.27	0.26	0.37	0.22	0.20
South	0.94	0.52	0.12	0.44	0.73	0.56

TEAC: Trolox Equivalent Antioxidant Capacity

For the free radicals $ABTS^{\bullet+}$, the antioxidant activity is less potent than that vis-à-vis DPPH, it reaches only a maximum of 51.49%, for 'Meski' leaves collected from the center of the country area. Aruoma (2003) noted the need of using several methods to understand the antioxidant activity of the tested samples. Ozcelik *et al*. (2003) reported that DPPH absorbance at 517 nm decreased by light, oxygen, pH and type of solvent in addition to the antioxidant.

'Meski' constitute a drought-tolerant cultivar. To protect against oxidative stress induced by the severity of climate, 'Meski' leaves continuously activated the antioxidant defense systems in the plants (Panday *et al*., 2017). It should have a better antioxidant system to effectively prevent oxidative damage and neutralized radicals (Rout and Shaw, 2001). This activity could be attributed to the richness in bioactive compounds such as phenolic compounds which is involved in the protection against oxidative stress caused by environmental conditions (Grace, 2005).

Correlation between bioactive compounds and antioxidant activity

Pearson correlation (r) was studied to determine the relationship between the potential antioxidant (IC50) and total polyphenol, O-diphenols, flavonoids, tannin, phytosterol, saponin and carotenoids levels of the extracts (Table 4). The antioxidant activity (IC50) was negatively and significantly correlated with total polyphenol (r = -0.997), flavonoids(r = -0.715), tannin (r = -0.998), phytosterol (r = -0.813), saponin (r = -0.974) and carotenoids (r = -0.924); whereas, chlorophyll pigments showed a positive and significant correlation, suggesting that the lower values of IC50 proved high antioxidant activities. Also, Table 4 showed a significant positive correlation between total polyphenol and tannin (r = 1), phytosterol (r = 0.76), saponin (r = 0.95) and carotenoids (r = 0.9). The data showed that these bioactive compounds contributed to increase the antioxidant activity. The study of Lee *et al*. (2009) showed a high correlation of the antioxidant activity with the amount of phenolic compounds and flavonoids in olive leaves. Many previous studies have reported significant correlation between polyphenolics and antioxidant activities in fruits, barley and mushrooms (Goupy *et al*., 1999; Leong and Shui, 2002; Choi and Wang, 2005). The observation agrees with other studies (Brahmi *et al*., 2012) which indicated that high polyphenolic content in extracts was significantly associated with antioxidant activity. Also, Kolo (2016) showed a relationship between sterol biosynthesis, sterol content, in maize leaves, and reactive oxygen species signalling.

The synergism between antioxidants (total polyphenol, flavonoids, tannin, phytosterol, saponin and carotenoids) could explain why the leaves' antioxidant capacity is higher than the individual content in each antioxidant. Similar results have been found in other researches (Thaipong *et al*., 2006; Silva and Sirasa, 2018).

Olive leaf extracts exhibited high antioxidant capacity which suggests olive leaf extract is effective in the function of scavenging free radicals (Luo *et al*., 2011). Olive leaves are a strong source of radical scavengers that are available throughout the year and all the types of leaves expected in an olive tree shoot can be exploited for the extraction of bioactive compounds, feasibly (Papoti and Tsimidou, 2009; Kolo, 2016).

Table 4. Pearson's correlation coefficients among the main phenolic compounds, pigment content, phytosterol and the antioxidant activity of olive leaves from Northern, Center and Southern areas

	Total phenol	Flavon-oids	Tan-nins	O-diph-enol	Phytos-terol	Saponin	Carote-noids	Chloro-phyll a	Chloro-phyll b	Total Chlorophyll	IC50
Total polyphenol	1	0.66^{ns}	1**	0.51^{ns}	0.767*	0.954**	0.903**	-0.87**	-0.928**	-0.929**	-0.997**
Flavonoids		1	0.67*	-0.14^{ns}	0.988**	0.988**	0.858**	-0.917**	-0.740*	-0.74*	-0.715*
Tannin			1	0.505^{ns}	0.778*	0.959**	0.907**	-0.876**	-0.929**	-0.93**	-0.998**
O-diphenol				1	-0.013^{ns}	0.303^{ns}	0.324^{ns}	-0.183^{ns}	-0.505^{ns}	-0.435^{ns}	-0.467^{ns}
Phytosterol					1	0.924**	0.917**	-0.960**	-0.822**	-0.868**	-0.813**
Saponin						1	0.966**	-0.966**	-0.936**	-0.957**	-0.974**
carotenoids							1	-0.982**	-0.947**	-0.97**	-0.924**
Chlorophyll a								1	0.917**	0.951**	0.899**
Chlorophyll b									1	0.995**	0.936**
Total chlorophyll										1	0.941**
IC50											1

Principal component analysis (PCA)

All collected data were submitted to principal component analysis (PCA) to better understand the changes of the phenolics, pigments, phytosterol and saponin contents of the studied leaves as function of various areas. The mean values of these compounds were used to build the PCA. Two principal groups concerned, with antioxidants compounds concentrations, were composed according to the PCA analysis.

The results showed a significant difference between Northern and Southern 'Meski' leaves on the level of total polyphenols, flavonoids, tannins, carotenoids, saponin and phytosterol compounds. The two first principal components (F1 and F2) explained 97.11% of the variance being F1 with 83.93% and F2 with 13.18%. Total phenols, flavonoids, tannins, carotenoids, saponin and phytosterol of Southern leaves were selected positively according to F1 axis. Although, total chlorophyll, chlorophyll *a* and *b* were elected negatively to the axis F1, suggesting that these pigments were negatively correlated to the other antioxidant's compounds.

According to PCA, the response of 'Meski' cultivar to climatic variability was different. In Southern area, the production of total polyphenol, flavonoids, tannins and the accumulation of carotenoids, phytosterol and saponin increased in leaves. Although, Northern leaves produced more chlorophyll *a*, *b* and total chlorophyll than the Southern ones. This result agreed with those of Khaleghi *et al*. (2012) and Cetinkaya *et al*. (2016) who showed that the water deficit induced a reduction of chlorophyll concentration in olive leaves, suggesting the sensitivity of this pigment to increase environmental stress. Since, the decreases of this pigment constitute a typical symptom of oxidative stress. Also, the study noted that the severity of climate positively affected the concentration of phenolic compounds, carotenoids, saponin and phytosterol, reduced total chlorophyll and increased the antioxidant capacity of leaves to shrink the effect of the environmental stress. This decline may be explained with the defense-related functions of phenolic compounds (Cetinkaya *et al*., 2016).

Identification and quantification of chemical compound of leaves extract by GC-MS

The analysis of leaves extract by CPG- FID and GC-MS of 'Meski', grown in various areas of Tunisia, was illustrated in Table 5. The results showed that 53.44, 59.4 and 55.39% of compounds were identified in northern, in Center and in southern leaves of 'Meski' cultivar respectively. Seventy-three compounds of the olive methanolic extracts were identified in leaves from various origins, such as phenolics, hydrocarbons, terpenoids, carbonylic compounds, fatty acids, cetones and esters.

Table 5. Changes of biochemical compounds in olive leaves extracts according to various origins

Compounds	Retention time (min)	Northern leaves	Center leaves	Southern leaves
Phenolic Compounds				
hydroxymethylbenzene	11.53	**6.58**		
α-ionene	11.87			**6.75**
trimethyl-tetrahydronaphthalene	11.88		0.26	
Benzaldehyde	12.50	1.55	3.52	**5.28**
Naphthalene, 1,2,3,4-tetrahydro-1,5,8-trimethyl-	14.33			1.13
1-(2,4,6-Trimethylphenyl)buta-1,3-diene	17.57			0.56
Benzene,2-(1,3-butadienyl)-1,3,5-trimethyl-	17.59	0.17		
3-Fluorocatechol	17.70			0.3
Guaiacol	17.79	1.48	2.4	3.07
Benzeneethanol	18.60		0.92	
4-Methyl-2,6-di-tert-butylphenol (BHT)	18.76	2.22		
Phenol, 2-methyl-	19.92	2.61		
Phenol	19.95	1.51		0.42
2-(t-butyl)benzothiophene	20.07			0.26
4-vinyl guaiacol	22.53	2.18	**7.77**	**5.32**
2,6-Dimethoxyphenol	23.38	0.31		0.94
Phenol, 2,5-bis(1,1-dimethylethyl)	24.02			0.17
N,N'-di-sec-Butyl-1,4-benzenediamine	24.03	0.29		
1-6-dimethylamino-2-phenyl-3-methyl(l-7-methoxynaphthalene)	24.48	1.71	3.94	1.81
2-(dimethylamino)-3-phenylbenzo[b] thiophene	27.36	0.35		
5-Methylthio-3-(2-(3,4,5-trimethoxyphenyl) ethenyl) pyrazole	28.79	0.62	4.09	
3,5'-biacenaphthene	29.37	0.89		2.47
Penoxaline	30.86			**15.32**
Naphthalene, 1-phenyl-4-(1-phenylethenyl)-	31.23		1.21	0.37
1-Phenyl-4-(1-phenylethenyl) naphthalene	33.59	4.44	4.4	0.38
Total		**26.3**	**28.51**	**44.55**
Hydrocarbons				
Dichloromethane ion	3.32		0.75	
Octamethyl-cyclotetrasiloxane	3.85			0.67
2-Hexyne	9.52	0.25		
1,4-Pentadiene, 2,3,3-trimethyl-	12.05		0.38	
5-Tetradecen-3-yne, (Z)	12.84	1.29		
Dimethylsulfoxide(DMSO)	13.48	2.51		1.57
Cycloheptane	16.39		0.63	
2-Methyl-1-octene	18.28		0.75	
2-methyl-2-ethenyl-1,1- difluorocyclopropane	19.69	0.17		
3,5'-biacenaphthene	33.90	1.00	1.39	1.24
10-Demethylsqualene	38.40	9.35		
Total		**14.57**	**3.27**	**4.11**
Terpenoids				
Oxygenated hemiterpen				
Oxirane, 2-ethyl-2-methyl-	14.25	0.34		
2-Furanmethanol	14.59	0.84	3.54	1.15
3,4-Dihydropyran	16.27	0.21	0.36	0.24

2(3H)-Furanone, 5-methyl-	16.41	0.38		
Monoterpen				
(2-Methylprop-1-enyl)-cyclohexa-1, 5-diene	20.50			0.55
Sesquiterpen				
5-ethyl-1,3-dimethylindan	15.74			0.24
Total		**1.77**	**3. 9**	**2.18**
Carbonylic compounds				
Furfural=2-Furancarboxaldehyde	11.29	1.28	8.73	3. 73
3-Cyclohexene-1-carboxaldehyde	23.79	2.43		
Total		**3.71**	**8.73**	**3.73**
Fatty acids				
Palmitic acid	23.11	1.21	1.82	1.52
Total		**1.21**	**1.82**	**1.52**
Cetones				
4-(Diethylamino)-2-butanone	7.87	0.37		
Bicyclo [3.3.0] oct-1(2)-en-3-one	15.03	4.78		
Corylon=Cyclotene	17.28	1.15	0.92	0.64
Ethanone, 1-(1,4-dimethyl-3-cyclohexen-1-yl)-	17.98		0.53	
Maltol	19.28		0.30	
Furaneol	20.19		0.77	0.46
1,3-Dimethylbicyclo[3.3.0]oct-3-en-2-one	21.28		0.82	
Megastigmatrienone 4	22.81			1.86
4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	23.29		0.64	0.37
1-(2'-Norbornylidene) propan-2-one	23.91			4.56
2,6-Dimethyl-.delta.1-bicyclo[4.4.0]decen-8-one	23.92			0.3
3-Hydroxy-beta.-damascone	26.82	0.38		0.92
Bicyclo[3.2.0]hept-6-e n-2-one, 1-propyl-	26.95		2.48	
1,3-Cyclopentanedione, 2-(2,2-dimethylpropylidene	28.30		9.47	
Dihydrojasmone	29.79		1.12	
Total		**6.68**	**17.05**	**8.19**
Ester				
Hexadecanoic acid, methyl ester	13.19	0.19		0.21
Total		**0.19**		**0.21**
Others				
Dimethylsiloxanepentamer	5.40	0.22		
vitispirane	12.77		0.82	
5-Methyl-2-furfural	13.31			1.05
1H-Pyrazole, 3-methyl-	16.00		0.26	
N-Formylpiperidine	16.75	0.4		
Ethanone, 1-(2-furanyl)-	17.28			0.28
Methyl n-amyldisulfide	23.52	0.84		
Total		**1.46**	**1.08**	**1.72**
Total Identified Compounds (%)		**59.73**	**68.26**	**66.21**

Note: The analysis of leaves extract was realized by CPG- FID and GC-MS of Meski, grown in various areas of Tunisia

The content of phenolic compounds in leaves extract increased with the severity of climate from the North (26.3%) to the South (44.33%). The level of Guaiacol (3.07%), Benzaldehyde (5.28%) and Phenol, 2,5-bis (1,1-dimethylethyl) increased in Southern leaves. These phenolic compounds could increase draught tolerance in leaves extract, while α-ionene (6.75%) and penoxaline (15.32%) accumulated only in Southern area, which seemed to be an indicator of drought stress. The results are in agreement with those of Bacelar *et*

al. (2006) who suggested that under environmental stress conditions, the production of total phenols in leaves increased.

Terpenes accumulated in higher quantity in Southern leaves of 'Meski' than in Northern ones. This result is in agreement with other studies and explained that terpene concentrations have been generally found to increase in drought conditions (Kainulainen *et al*., 1992; Llusia and Penuelas, 1998; Blanch *et al*., 2009). Also, Flamini *et al*. (2003) showed that aldehyde and terpene compositions changed significantly according to season in the leaves of an Italian olive cultivar. These secondary metabolites are reported to possess antioxidant potentials (Yasukazu and Takuma, 2008) and play a primordial role in the adaptation to surround the severity of the climate.

Conclusions

The present study showed a significant influence of the climate on the bioactive compounds content and the antioxidant activity of 'Meski' olive trees' leaves. To surround the aggression of climate and to provide tolerance to stress, olive tree 'Meski' increased phenolic compounds, flavonoids, tannins, phytosterols, saponin and carotenoids contents and decreased their chlorophyll levels in trees' leaves cultivated in Southern areas. The highest level of antioxidants compounds in Southern leaves could be contributed to increase the capacity of leaves to scavenging free radicals and to reduce the oxidative stress. Thus, it can be concluded that the biosynthesis and regulation of these secondary metabolites can be considered as a response of the tree to oppose the oxidative stress that results from the severity of climatic conditions, characteristic of the Southern area. Also, it is considered as an adaptation to the tree to survive and tolerate environmental stress.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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