Determination of some biochemical parameters from leaves of *Quercus ilex* L.(Fagaceae), collected in Djabel Zagouan (Tunisia)

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Abstract

Biochemical characterization, such as, fatty acids, essential oils composition, polyphenols content and antioxidant activities, were investigated from leaves, of *Quercus ilex* L.(Fagaceae), collected in DjabelZagouan (Tunisia). Only the fatty acid composition was determined by gas chromatography after transformation of the fatty acids into methyl ester derivatives compatible for injection in a Gas Chromatograph,and determined by gas chromatography (GC-FID). Essential oils were extracted by hydrodistillation and, analyzed by gas chromatography (GC-FID). Total phenolic content, concentration of flavonoids and,*in vitro,* antioxidant activities were determined, using spectrophotometric methods. The antioxidant activities was tested by the DPPH (2, 2-diphenyl-1-picrylhydrazyl), free radical scavenging assay.

Results showed that total fatty acid content varied from 1.78 to 4.86%, based on dry weight (dw) and the predominants fatty acids were oleic acid (63.43–59.76%), linoleic acid (14.93–16.05%) and palmitic acid (9.16–12.13%). The oils contain, an appreciable, amount of unsaturated fatty acids (73.86–85.59%).This study showed that Holm oak leaves, are rich in an unusual fatty acid, the oleic one. The essential oil yield varied, from 0.01 to 0.04%. So the major components, in leaves oils from *Quercus ilex* L., were *trans*-2-hexanal (14.89–16.93%), viridiflorol (12.94–15.24%) and sabinene (6.84–9.65%).The total phenolic, content of the samples, varied, from 2.93 to 3.40 mg/g of dry weight, expressed as gallic acid equivalents.

The total flavonoïd concentrations varied, from 1.83 to 2.72 mg/g, of dry weight quercetin equivalents. The extracts of the leaves were observed displaying strong DPPH free radical scavenging action, with IC_{50} value, ranged, from 102.66 to 134.83 µg/mL, comparing to the IC_{50} value (48.31µg/mL), of reference standard. These results suggest that phenolics, in this species, provide substantial antioxidant activity and *Quercus. Ilex* L. may be a potential source of natural antioxidants.

Keywords: antioxidant activity, Djebel Zaghouan, essential oils, fatty acids, *Quercus ilex* L.

INTRODUCTION

Vegetable oils and fats are used in the food industry, for the manufacture of a wide variety of products, ranging from margarines to chocolate, or used directly, as salad and cooking oils [1].

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Secondary metabolites constitute biologically and chemically, interesting, group of substances, extracted from the plant kingdom. Essential oil, of plants, shows many biological activities, in addition to their use in food, flavor, perfumery, cosmetic and pharmaceutical industries, as natural antioxidants [4,5,6]. Essential oil has been used, since ancient times, for medicinal purposes and known for its anti-rheumatic, anti-inflammatory and antispasmodic properties [7]. It has demonstrated,as powerful antimutagenic, antibacterial and it posed chemopreventive properties [8,9].For the secondary metabolites grouped, essential oil imparts the much needed curative properties [10]. Different studies, made on the essential oil, show influence of the area of culture, of the variety and the harvest season, on the chemical composition [11-13]

The presence of phenolic compounds, in herbs and spices, along with the essential oils, is gaining increasing attention, because of their various functions, such as antioxidant activity and flavouring properties [14]. Indeed, natural bioactive compounds, like phenols and flavonoids, are the important secondary metabolites in plants, having intrinsic properties, that affect appearance, taste, odor and oxidative stability of plant, based foods[15]. Secondary metabolites, from plants, have important biological and pharmacological activities, such as anti-oxidative, anti-allergic, antibiotic, hypoglycemic and anti-carcinogenic activities [16].

The genus *Quercus* L. (Fagaceae) comprises more than 450 species and it is one of the most important genera in the family, from several aspects, such as wide distribution, notable bio-ecological features, great economic benefits[17].Many species of the *Quercus* are very characteristic of the Mediterranean area [18]. The genus *Quercus* L. has been of interest to researchers, due to its important usage in folk medicine, in agro-alimentary, in the maturation of wines in oak barrels, in wood industry and consumption as food, for both humans and animals (Kariotiet *al.*,2010 ;Söhretoğluet*al.*, 2012).

Holm oak (*Quercus ilex*L.) is one of the most important and widespread elements of the vegetation communities, in the western Mediterranean Basin (Baldantoniet *al.*, 2011 ; Custódioet

al., 2013).The constituents of the essential oil of *Quercus. Ilex*L., fromPosedarje,were identified by Peifhofer (1998). He found eighty-fivecompounds, with dominant constituents being *trans*-2-Hexanal, Viridiflorol, *β*-Bourbonene, Hexanal, Oxygenated sesquiterpene and Aromadendrene. However, so far merely a few species of oak were studied, as regards the essential oil of the leaves:*Quercusagrifolia* Née (Palma-Flaming andKepner, 1983) and *Quercusrobur* (Engel et al., 1993). Holm oak (*Quercus. ilex* L.) was shown to emit large amounts, of monoterpenes and traces of isoprene (Staudtet *al.,* 2001, Fischbach et *al.,* 2000).

The major fatty acids in acorns of *Quercus ilexand Quercusrotundifolia*trees, from Italy and Spain, were oleic acid (40-70%), linoleic (16.5-17%), palmitic (12.1-13%). Stearic acid and linolenic acid, were found in small amounts (1-5%);myristic, palmitoleic, arachidic, 11-eicosenoic, and behenic acids, were found, in amounts, below 0.5% or in traces (Rafiiet *al.,* 1991). *Quercus*(oak) species,are a rich source of phenolic compounds, such as proanthocyanidins, tannins and acylated flavonoid glycosides(Kariotiet *al.,*2009, Söhretoğluet *al.,*2012). Species, of the genus *Quercus*, have been, for long, used in traditional medicine, as haemostatic, in the treatment of gastrointestinal disorders. Polar extracts, of the leaves, have shown antibacterial and antiinflammatory activities, attributed to their high phenolic content(Kariotiet *al.,*2009). It has been previously reported that the methanol extracts, of *Quercus ilex* leaves, from Turkey and *Quercusrobur* bark, possess antibacterial activity, which can be related to the richness, of *Quercus* species extracts, on phenolic compounds, such flavonoids and tannins (Berahouet *al.,* 2007, Hosamet *al.,* 2019).The antioxidant and antibacterial potential, of several *Quercus* alcoholic extracts, has set under discussion their application, in food industry, as antioxidants, for the more efficient conservation of foods and, also, for their beneficial role in human health (Berahouet *al.,* 2007, Karioti et *al.,* 2010).The growth and function of plants, in the ecosystems, are influenced by different factors, such as the

species, climate, soil, altitude, and geographical area.Each factor of these has a substantial effect on the quantity and quality (Mahdaviet *al.*, 2013).

The current study is a detailed investigation of the variation of fatty acids, essential oils composition, polyphenols and antioxidant activity, among *Quercus ilex*, collected from three sites, in mount Zaghouan (Tunisia). The detected variability will be interpreted and discussed according to environmental conditions and the impact of the altitude of the studied sites.

MATERIALS AND METHODS

Sites and Plant material

Leaves of Holm oak (*Quercus ilex* L.) were collected from three sites, in the "Park of DjebelZaghouan", located in the northeast of Tunisia: site 1(36°22'09,287"N-10°06'26,138"E); site 2 (36°22'18,696"N-10°06'32,655"E) and site 3(36°22'27,273"N-10°06'48,367"E), with sub-humid bioclimatic stage(Table 1). The average annual rainfall is between 500 and 650 mm / year.

Table 1. The characteristics of different sites from leaves of *Quercus ilex* L.

Sample site	Characterizations			
	Latitude	Longitude	Altitude	Environment
Site 1	36°22'09,287"N	10°06'26,138" (E)	730 m	preserved environment where the plants are vigorous producing a very large biomass.
Site 2	36°22'18,696" (N)	10°06'32,655" (E)	813m	moderately degraded environment where plants show an average force
Site 3	36°22'27,273" (N)	10°06'48,367" (E)	831 m	degraded environment where plants produce small biomass and trees are stunted

The harvested materials were air-dried at room temperature (20±2°C), for one week, ground in Retsch blender (Normandie-Labo, Normandy, France), sieved through 0.5 mm mesh screen, to obtain an uniform particle size .

Chemical analysis

A set of 24 fatty acid methyl esters (FAMEs), reference standards, were obtained from Fluka (Steinheim, Germany). Chloroform, methanol and *n*-pentane, were purchased from Lab Scan (Lab Scan, Ltd., Dublin, Ireland). Sodium bicarbonate, was purchased from Chemi-Pharma (Chemi-Pharma, Le Bardo, Tunisia). Diethyl ether,*n*-hexane and methanol, were obtained from Baker (J.T. Baker, Daventer, Holland). Anhydrous magnesium sulfate, was obtained from Panreac (Panreac, Barcelona, Spain). Sodium methoxide,was obtained from Sigma–Aldrich (Sigma-Aldrich, Buchs, Switzerland). Folin-Ciocalteu reagent, gallic acid, quercetin, Ascorbic acid, $AlCl_3$and 2, and 2-Diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma–Aldrich (St. Louis, MO). All solvents and reagents used, were of the highest purity.

Total lipids extraction and Fatty acid methylation

Samples of ground powder (1g), in triplicate, were weighed and extracted, using the modified method of Bligh and Dyer (1959). A chloroform/methanol mixture (2:1, v/v), was used for total lipid extraction. The mixture was shaken and centrifuged (Eppendorf 5810R, Le Pecq, France) at 3,000 × g for 10 min, to allow phase development. The organic layer, containing total lipids, was collected and filtered. The total extracted lipid material, was recovered, after the solvent was removed, in a stream of nitrogen. The total fat content (TFC), expressed as percent of the dry matter, was calculated using the following formula (Gandouret *al.*, 2011):

$$\mathbf{TFC\ (\%\ of\ dw)} = \mathbf{100}\frac{\mathbf{m}}{\mathbf{M}} \quad (1)$$

Where **TFC** is the total fat content, **m** the mass of extracted oil (mg), **M** the mass of dry matter (mg) and **dw**the dry weight.

Total fatty acids were converted into their methyl esters, using 3% sodium methoxide in methanol, according to the method of Cecchi et *al.* (1985). Methyl nonadecanoate (C19:0), was used as internal standard. The fatty acids methyl esters, (FAMEs), obtained, were subjected to GC analyses.

Fatty acid gas chromatography analysis

The fatty acid methyl esters (FAMEs) were analysed by gas chromatography, using a Shimadzu HRGC-2010 chromatograph (Shimadzu Corporation, Kyoto, Japan), equipped with flame ionization detector (FID), Auto-injector AOC-20i and auto-sampler AOC-20s. Separation of different FAMEs, was performed on a polar capillary column, type TRB-Wax (30 m × 0.25 mm, 0.25µm film thickness). The oven temperature was programmed, as follows: starting from 150°C (5 min), increasing to 200°C, at a rate of 10°C/min, held for 5 min, then ramped to 240°C, at a rate of 15°C/min, and finally held, for 5 min. The injector and detector temperature, were 250 and 275°C, respectively. The split ratio, was 1:100 and the injection volume, was 1µL. All samples were analyzed in three replications.

Identification of FAMEs, was made by comparing their retention times, with those of reference standards. The FAMEs composition (percent) refers to the percentage ratio, of each component to total fatty acid.

Essential oil isolation

The air-dried materials (100 g), were hydrodistilled, for 2h, in 2L distillation flask, a condenser and a receiving vessel. The obtained distillate, was extracted, using diethyl-ether as solvent (v/v) and dried over anhydrous sulphate sodium. The organic layer, was then concentrated at 35°C, using a Vigreux column and the essential oil, stored at –4°C prior to analysis. The essential oil yield, was estimated, according to dry leaves matter, by using the following equation (Sangunet *al.*, 2007).

$$\mathbf{Rhe(\%) = 100\frac{Mhe}{Ms}} \quad (2)$$

Where M_{he} = essential oil mass (g), M_s = dry leaves matter mass (g), R_{he} = essential oil yield (%).

Essential oil gas chromatography analysis

Gas chromatography analyses, were done with Shimadzu HRGC-2010 gas chromatograph (Shimadzu Co, Kyoto, Japan), equipped with flame ionisation detector (FID), Auto-injector AOC-20i and auto-sampler AOC-20s. An apolar column Rtx-1 (30 m x 0.25 mm, 0.32 µm film thickness), was used. The oven temperature, was held at 50°C, for 10 min, then programmed at 2°C/min to 190°C, then held isothermal, for 10 min. The injector and detector temperature, were programmed at 230°C. The flow of the carrier gas (N_2), was 1.6 ml/min and the split ration, was 1:20. Injection volume, for all samples, was 0.5µl of diluted oils in *n*-pentane (LabScan Dublin, Ireland). The volatile compounds, were identified by comparison of their retention indices (RI) relative to(C_7–C_{20}) *n*-alkanes, with those of literature and/or with those of authentic compounds, available in our laboratory. Relative percentage amounts of the identified compounds were obtained from the electronic integration, of the FID peak areasin the chromatogram.

Preparation of methanolic extracts

Methanolic extracts, of *Quercus ilex* L., was obtained, as described by Eghdami and Sadeghi (2010), with slight modification. From the dried powdered samples in triplicate, 1g were weighed and put into 100 mL flasks. Each flask was added with 20 mL of 80% methanol. After 48h of storage, at room temperature and under stirring, the supernatants, were filtered and these filtered extracts, were dried at the temperature of 30°Cby the oven. The dried samples, were, then, weighed. The extracted samples, were, then, dissolved in 3mL methanol and stored at +4°C, until analysis (Benhammouet al., 2008).

Total phenolic content

Total phenolics were determined with Folin–Ciocalteu (F-C) assay, as per Singleton et *al.*, (1999) method, slightly modified by Dewantoet *al.*, (2002). Briefly, 0.125 mL diluted extract, was added to 0.5 mL deionized water and 0.125 mL (F-C) reagent. After shaking, the mixture was incubated for 3 min, at room temperature. Then 1.52 mL of sodium carbonate solution (7%), was added. The volume obtained, was adjusted to 3 ml, using distilled water, mixed vigorously,

and held for 90 min, at ambient temperature. The absorbance of solution, was, then, measured at 760 nm, using a UV/Vis Jenway 6300 spectrophotometer (Jenway Ltd., United Kingdom), against a blank. The total phenolics content, was expressed, as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW), through the calibration curve of gallic acid. All measurements were performed in triplicate.

Total flavonoids content

Total flavonoids content, were determined, according to the aluminium chloride colorimetric method (Djeridaneet *al.*, 2006). One milliliter of diluted methanolicextract, was mixed with 1.0 mL of 2% $AlCl_3$methanolic solution. After incubation, at room temperature for 15 min, the absorbance, was measured at 430 nm, using a UV/Vis Jenway6300spectrophotometer (JenwayLtd.,United Kingdom). The total flavonoids content, were calculated on the basis of the calibration curve of quercetin, and were expressed, as mg quercetin equivalents per g dry weight (mgQE/g DW). All samples were analyzed in three replications.

Antiradical scavenging activity by DPPH method

Plant extracts, were tested for the scavenging effect on DPPH radical, according to the method described by Bracaet *al.* (2001).Briefly, 0.2 ml, of extract sample, at various concentrations (10–250 µg/mL), was added to 5 ml of 0.004% (w/v) stock solution of DPPH, in methanol (80%). An equal amount of water, was added to the control. After 30 min incubation, at room temperature, the absorbance was read, against a blank at 517 nm, in a UV/Vis Jenway-6300 (JenwayLtd.,United Kingdom). All the analyses, were done in three replicates. Ascorbic acid was taken, as the standard. The DPPH radical scavenging activity, in terms of percentage, was calculated, according to the following equation (Kumaranet*al.*, 2006):

$$\boldsymbol{I(\%) = [(A0 - A1)/A0]\ x100}$$(Orsavova et al., 2015)

Where*I* was DPPH inhibition (%), $\boldsymbol{A_0}$was the absorbance of the control, and $\boldsymbol{A_1}$ was the absorbance of the extract/standard.

The concentration of sample, required for 50% inhibition, was determined and represented as IC_{50} for each of test solution, which is expressed as µg/ml. All measurements were performed in triplicate.

Data analysis

All the analyses were performed in triplicate, for each sample and expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test, was applied, to compare means, at the significance level $p < 0.05$. All analyses were performed, using SAS v 9.1 software package.

RESULTS AND DISCUSSION

Total lipid content

The amounts, of total lipids in *Q. ilex* leaves, are presented in table 2. The lipid contents, of leaves, varied significantly ($P<0.05$) and ranged from 1.78 to 4.86%, based on dry weight (dw). The lipid content was highest in samples of *Quercus ilex*L., collected in site1 (4.86%dw) and lowest in samples, collected in site 3 (1.78 % dw).The samples, collected in the site 1 and in site 2, were outstanding, with their markedly higher total lipid content, with values 4.86 and2.56 mg/g dw, respectively. These variations, in total lipids content, among ecotypes, could be mainly due to the effect of genetic factors, as well as environmental, edaphic and cultural conditions (Uzunet *al.*, 2002, Ravi et *al.*, 2007).

Fatty acid composition

A statistically significant($P < 0.05$) variation, was determined, in the Fatty acid composition,between the samples, collected in the differents sites. The saturated and unsaturated fatty acid compositions of *Quercus ilex*L.samples, are given in Table1. Eleven fatty acids, were identified, for the Holm oak samples and their composition was evaluated, for differentssamples. Total saturated fatty acids (SFA),was ranged between 14.41 and 26.14 % (Table1). While palmitic acid (9.16–12.13 %,) and stearic acids (3.60–5.25%), were dominant saturated fatty

acids in all samples. However,myristic acid (0.35–2.94%), behenic acid (0.42–2.10%) and lignoceric acid (0.11–2.85%) were found in lowest quantity.Total unsaturated fatty acids (USFA),was ranged between 73.86 and 85.59% (Table 2). Five unsaturated fatty acids, were identified and quantified among which oleic acid (C18:1n9), contributed and ranged, between 53.54 and 63.43 % to total fatty acid, followed by linoleic acid (C18:2n6), ranged between 14.48 and 16.05% to total. The remaining three unsaturated fatty acids contributed only, ranged between 0.73 and 3.33% (Table2).

Table 2. Total lipid content and fatty acid composition (%) of leaves of *Quercus ilex* L., determined in three collection sites, in preserved state (Site 1), in moderately degraded state (Site 2) and in degraded state (Site 3), of National Park Jebel Zaghouan

Fatty acids	Site1	Site2	Site3
Total lipid content(% dw)	4.86±0.18^{a}	2.56±0.80^{b}	1.78±0.25^{c}
Myristic acid (C14:0)	0.35±0.02^{b}	2.94±0.11^{a}	2.64±0.37^{a}
Palmiticacid (C16:0)	9.16±0.12^{c}	10.02±0.18^{b}	12.13±0.20^{a}
Palmitoleicacid (C16:1)	0.73±0.03^{b}	0.38±0.01^{b}	1.84±0.05^{a}
Margaricacid (C17:0)	0.77±0.03^{b}	1.24±0.06^{a}	1.17±0.08^{a}
Stearicacid (C18:0)	3.60±0.15^{b}	4.42±0.37^{b}	5.25±0.06^{a}
Oleicacid (c9, C18:1)	63.43±0.46^{a}	59.76±0.50^{b}	53.54±0.49^{c}
Linoleicacid (c9, 12-C18:2)	16.05±0.22^{a}	14.93±0.17^{b}	14.48±0.19^{b}
Linolenicacid (C18:3n3)	3.33±0.06^{a}	2.40±0.03^{b}	2.22±0.05^{b}
Eicosenoicacid (c11, C20:1)	2.05±0.04^{a}	1.86±0.02^{a}	1.78±0.03^{a}
Behenicacid (C22:0)	0.42±0.01^{b}	0.95±0.03^{b}	2.10±0.01^{a}
Lignocericacid (C24:0)	0.11±0.07^{b}	1.10±0.05^{b}	2.85±0.03^{a}
SFA	14.41±0.17^{c}	20.67±0.35^{b}	26.14±0.32^{a}
MUFA	66.21±0.53^{a}	62.00±0.47^{b}	57.16±0.45^{c}
PUFA	19.38±0.11^{a}	17.33±0.16^{b}	16.70±0.12^{b}
USFA	85.59±0.72^{a}	79.33±0.87^{b}	73.86±0.99^{c}
SFA/ PUFA	0.74	1.19	1. 56

Values of the same line with different superscripts (a–c) are significantly different at p < 0.05.SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UFA: unsaturated fatty acids.

The fatty acid composition, of the leaf oils investigated, revealed oleic acid, as the predominant monounsaturated fatty acid (MUFA). The oleic acid (C18:1n9) content is as follows : 63.43%, for Site1 ; 59.76%, for Site2and 53.54%, for Site 3.The total MUFA made up the

highest proportion (57.16–66.21%), of the total fatty acids of samples, whereas the total SFA were small proportion.

The fatty acid composition, of Holm oak samples, revealed linoleic acid, as the predominant polyunsaturated fatty acid (PUFA). The percentages of linoleic acid (C18:3n2)are as follows : 16.05%, for Site 1 ; 14.93%, for Site 2 and 14.48% for Site 3. This preliminary study shows, that *Quercus ilex*L. oils contain high relative percentages of linoleic acid (Table 2). Indeed, linoleic acid is, undoubtedly,the one of the most important polyunsaturated fatty acids, in human food, because of its prevention of distinct heart vascular diseases (Omodeet*al.*, 1995).

Regarding the SFA/PUFA ratio, Site1 showed the lowest one (0.74), in comparison to Site 2 and to Site 3, which its value was 1.19 and 1.56, respectively.

The differences observed, between sites, for fatty acid composition, may be explained by the different altitudes of the sites, environmental conditions and by genotypic factors. In fact,it has been well established, by many authors, that fatty acid composition had been influenced by altitude (Beyhanet *al.*, 2011).

The Saturated and unsaturated acids are influenced by environmental conditions, such as temperature, rainfall, and genotypes (Sadeghi and Talaii, 2000). The amount, of fatty acids, on the genotypic, is one of the most influential factors (Uzunet *al.*,2002 ;Tulukcu, 2011). Different authors have observed, that fatty acid contents were significantly influenced by years, various physiological, geographical, ecological and cultural factors (Uzunet *al.*,2002;Msaâdaet *al.*, 2009, [1]. Indeed, the level, of this fatty acid, has been shown to be greatly influenced by the impact of abiotic conditions, such as water availability (Laribiet *al.*, 2011).

Yields and essential oils components

According to our results, the observed yield of essential oils increases significantly, from Site1 to Site3 (Table 3). In fact, the lowest yield of essential oil, based on dry weight, is recorded for the samples, collected in the Site3 (0.01%), while the highest level, is observed for the samples,

collected in the Site1, with a mean value of 0.04%.The yields of the Site1 and Site 2, are higher than that obtained with those of the *Quercus ilex*L., from Autria (Peifhofer, 1998). However, the yield of the Site 3 is the same as that, obtained by this author. Variation, in oil yield, can be attributed to some factors, like conditions of plant growth, environmental and region. The oil yield, during plant growth, is particularly susceptible to environmental conditions, such as light, nutrient availability, day length and daily temperature (Skoulaet *al.*, 2000,Msaadaet *al.*, 2009).

The essential oil compositions, of Tunisian *Quercus ilex* L., collected in three different Sites, were determined by GC–FID analysis. A total of 61 compounds, were identified, with a significant variation, of the essential oil chemical composition, according to studied sites (Table 3). The major components, in leaves oils from *Quercus ilex* L., were *trans*-2-hexanal (14.89–16.93%), viridiflorol (12.94–15.24%) and sabinene (6.84–9.65%). The results indicated that *Quercus ilex* L. samples, collected from Site1, had significantly the highest inhibition percentages, of the major components. The samples, collected from Site2, were significantly, the second, and those, collected from Site3, was the third (Table 3).

Major compounds, for the studied sites, were followed by others components, with lower percentages, which are hexanol (2.5–4.71%), para-Cymene (0.70–4.39%), α-Thujene (2.03–4.3%),aromadendrene (2.44–3.07%), β-Pinene(0.02–4.47%), α-Pinene (2.16–3.11%), palustrol (1.87–2.13%), nonanal (0.06–2.11%), linolyl acetate (1.44–1.85%), nerol(0.09–2.30%), α-cubebene (0.48–1.95%), carvotanacetone(1.62–1.92%), germacrene D (0.82–1.51%), carvacrol (0.24–1.94%), perillaldehyde (0.09–1.05%), carvacryl methyl oxide(0.96–1.81%), decanal(1.04–1.88%), β-Myrcene (0.08–3.07%), bornyl acetate(0.95–1.44%), α-Terpinolene(0.05–1.48%) and para-Cymenene (0.06–1.25%) (Table2).Our results are comparable to the previous study, regarding *Quercus ilex* L., from Autria (Peifhofer, 1998). According to the last author, leaves essential oils, were shown to be dominatedby*trans*-2-hexanal (18.06%), viridiflorol (14.84%), β-Boubonene (8.81%), oxygenated sesquiterpenes(3.51%), hexanal (3.21%) and aromadendrene

(2.73%). High amounts, of *trans*-2-hexanal, were, also, detected in leaves of *Quercusagrifolia* (Palma-Fleming andKepner, 1983) and in those of *Quercusrobur* (Engel et *al.*, 1993).

Table 3. Chemical composition of essential oils, obtained from leaves of *Quercus ilex* L., determined in three collection sites, in preserved state (Site 1), in moderately degraded state (Site 2) and in degraded state (Site 3), of National Park Jebel Zaghouan

Volatile Compound	RI	Percentage of component		
		Site1	Site2	Site3
Essential oil yield (% dw)		0.04[a]	0.02[b]	0.01[c]
Hexanol	831	4.71[a]	3.91[b]	2.51[c]
Nonane	901	0.59[a]	0.33[b]	0.29[b]
α-Thujene	922	4.30[a]	2.97[b]	2.03[c]
trans-2-Hexanal	925	16.93[a]	15.53[b]	14.89[c]
(z)-2-heptenal	926	0.93[a]	0.83[b]	0.82[b]
α-Pinene	930	2.16[b]	3.05[a]	3.11[a]
Camphene	941	0.15[b]	0.63[a]	0.06[b]
1-Octen-3-ol	959	0.05[c]	0.81[b]	0.97[a]
Sabinene	967	9.65[a]	7.21[b]	6.84[c]
β-Pinene	975	4.47[a]	0.57[b]	0.02[c]
β-Myrcene	980	1.12[b]	3.07[a]	0.08[c]
Decane	1000	0.22[c]	0.57[b]	0.97[a]
α-Terpinene	1006	0.05[b]	0.52[a]	0.49[a]
para-Cymene	1015	4.39[a]	4.11[b]	0.70[c]
γ-terpinene	1067	2.14[a]	1.89[a]	1.48[b]
para-Cymenene	1071	1.25[a]	0.22[b]	0.06[c]
Nonanal	1081	2.11[a]	0.06[c]	0.8[b]
α-Terpinolene	1089	1.48[a]	0.05[c]	0.85[b]
Undecane	1098	Tr	0.45[a]	tr
cis-para-Menth-2-ene-1-ol	1106	0.14[b]	0.71[a]	tr
2-phenylethanol	1119	Tr	0.13[b]	4.81[a]
trans-para-Menth-2-ene-1-ol	1123	0.07[a]	0.08[a]	0.03[b]
Camphre	1125	0.44[a]	0.13[b]	0.32[a]
Neryloxide	1137	0.08[a]	0.12[a]	0.09[a]
Isoborneol	1143	2.82[a]	2.05[b]	1.05[c]
Lavandulol	1148	0.06[b]	0.44[a]	0.06[b]
Borneol	1150	0.74[a]	0.14[b]	0.07[b]
Terpinene-4-ol	1163	0.14[a]	0.11[a]	0.06[a]
α-Terpineol	1176	0.39[a]	0.14[b]	0.07[b]
cis-Piperitol	1179	0.31[b]	0.39[a]	0.37[a]
Decanal	1182	1.88[a]	1.06[b]	1.04[b]
trans-Piperitol	1188	0.07[b]	0.23[a]	0.21[a]
Octylacetate	1191	0.07[b]	0.45[a]	0.07[b]

β-Cyclocitral	1197	0.05[b]	0.53[a]	0.06[b]
Fenchylacetate	1203	0.04[b]	0.64[a]	0.75[a]
Nerol	1205	0.09[c]	2.30[a]	0.84[b]
Pulegone	1214	0.06[a]	0.05[a]	0.03[a]
Thymylmethyloxide	1215	0.06[c]	0.97[b]	1.48[a]
Carvotanacetone	1219	1.92[a]	1.65[b]	1.62[b]
Carvacrylmethyloxide	1224	1.81[a]	1.44[b]	0.96[c]
Carvacrolmethylether	1226	0.37[a]	tr	0.17[b]
Linolylacetate	1236	1.59[b]	1.44[c]	1.85[a]
Linalylacetate	1240	0.15[b]	0.46[a]	0.05[c]
Perillaldehyde	1245	0.09[b]	1.03[a]	1.05[a]
Decanol	1253	0.23[c]	0.49[b]	0.75[a]
Thymol	1267	Tr	0.53[b]	0.82[a]
Bornylacetate	1269	1.44[a]	0.98[b]	0.95[b]
Carvacrol	1279	0.24[c]	1.67[b]	1.94[a]
Pinocarvylacetate	1282	0.30[c]	0.93[a]	0.79[b]
Tridecane	1302	0.85[a]	0.74[b]	0.66[b]
α-Terpinylacetate	1328	0.12[a]	0.06[b]	0.08[b]
Nerylacetate	1340	0.46[b]	0.96[a]	0.53[b]
α-cubebene	1350	0.48[c]	1.44[b]	1.95[a]
(E)-β-damascenone	1362	0.11[a]	0.06[a]	0.04[b]
(E)-β-Caryophyllene	1420	0.12[a]	0.11[a]	tr
Aromadendrene	1434	3.07[a]	3.05[a]	2.44[b]
β-Ionone	1462	0.12[a]	0.14[a]	0.13[a]
Germacrene D	1478	0.82[c]	1.51[a]	1.26[b]
γ-Cadinene	1507	0.06[b]	0.09[a]	0.05[b]
δ-Cadinene	1517	0.09[a]	0.07[a]	0.05[a]
α-Calacorene	1539	0.23[a]	0.10[b]	0.12[b]
Palustrol	1562	1.87[b]	2.13[a]	2.15[a]
Caryophylleneoxide	1575	0.07[b]	0.22[a]	0.06[b]
Viridiflorol	1579	15.24[a]	14.47[b]	12.94[c]
Ledol	1585	0.18[a]	0.15[b]	0.14[b]
Heptadecane	1705	Tr	0.86[a]	0.79[b]
Nonadecane	1900	0.24[b]	0.26[a]	0.33[a]
(E)-Phytol	2102	0.25[b]	0.53[a]	0.60[a]
Tricosene	2300	0.29[a]	0.28[a]	0.18[b]
Chemical classes				
Monoterpenehydrocarbons (%)		46.84[a]	39.61[b]	30.55[c]
Oxygenatedmonoterpenes (%)		13.86[c]	18.77[a]	15.46[b]
Sesquiterpenehydrocarbons(%)		6.52[b]	6.92[a]	6.15[c]
Oxygenatedsesquiterpenes(%)		22.98[c]	24.82[b]	25.97[a]
Aldehydes (%)		4.92[a]	1.95[c]	2.66[b]
Others (%)		1.90[b]	3.22[a]	3.04[a]

Total identified (%)	97.02[a]	95.30[b]	83.83[c]

RI: Retention indices on the apolar Rtx-1 column; tr: (<0.01%). Values with different superscripts in the same line are significantly different at $p < 0.05$.

The chemical class characterization, of Holm oak leaf essential oil, showed the prevalence of monoterpene hydrocarbons (30.55–46.84%). The oxygenated sesquiterpenes (22.98–25.97%), formed the second main class. The oxygenated monoterpenes (13.86–18.77 %), formed the third main class. The remaining fractions, such as aldehydes and sesquiterpene hydrocarbons, formed the minor essential oil chemical classes, of Holm oak leaves studied.

The samples, collected from Site1, showed high percentages of monoterpene hydrocarbons (46.84%). Moderate percentages, of monoterpene hydrocarbons, was found, in leaves of Site2 (39.61%) and the low levels of monoterpene hydrocarbons (30.55%), were found, in leaves of Site2. However, the samples, collected in the Site2, have higher content oxygenated monoterpenes (18.77%). On the other hand, the samples, collected in the Site2 and Site3, are high in oxygenated monoterpenes (25.97%).The samples, in these two sites, are significantly ($P<0.05$) rich in oxygenated sesquiterpenes, compared with the samples, collected in the Site1.

This variability can be explained by the influence of various factors. In fact, it was, previously, shown, for the majority of vegetal species, that primary and secondary metabolism are strongly influenced by plant physiology and genetic, by climatic and edaphic factors, by the harvest period, inducing changes in chemical qualitative and quantitative composition (Msaadaet *al.*,2009 ; Hosni et *al.*, 2011 ; Jemâa2014). Thus, the same species may show different chemical compositions, from a region to another, due to genetic variability, influenced by environmental factors (Pritchard et *al.*, 2000). The influence of environmental factors, in the chemical composition of essential oils, has, also, been reported in *A. absinthium*(Bailenet *al.*, 2013). Variations in the relative amounts of these compounds, under different environmental conditions, indicate their adaptively ecological value.

The variations observed can be explained, also, by the influence of the altitude. Indeed, Essential oils composition, of peppermint (*Menthapiperita*), was different because of the altitude and irregular daylight (Yazdaniet *al.,* 2002). Plants are grown and produced, in different ecosystems and sites, under the influence of different potential factors, including the altitude, as one of the vital determinants in the quantity and quality of the plants (Mahdavi and*al.,* 2013). Chemical components of the essential oils, are cited to vary qualitatively and quantitatively, according to geographical location and environmental conditions (Bakkaliet *al.*,2008 ;Oliveira et *al.,* 2013).

Kouyokhi and*al.* (2008), reported that phytochemical variations, were found, not only among samples from different regions, but also among samples from the same region, with different altitudes, reflecting the effect of environment on essential oil components.Habibi and*al.*,(2006), reported that, also, the essential of *Thymus kotschyanus* differ in different altitudes, but, as the altitude increases, the amount of essential oil dropped.

Independently of sample collection sites, essential oil, of *Quercusilex*L. leaves, is rich in moterpenes. Indeed,Fischbachet*al.*(2000), reported that leaves, of some oak species, as the evergreen sclerophyllous*Quercus ilex* L., emit large amounts of monoterpenes, despite the absence of storage pools. This monoterpene emission, is largely associated with photosynthesis, since it is stimulated by light, declines in dark, and is inhibited in the absence of CO_2 in air (Loreto et *al.,* 1996a,b).

Total Phenolic Content

Total phenolic values, of *Quercus ilex*L. leaves, are given in Table 4. The total phenolic content, varied widely, and ranged from 2.53±0.03 to 3.40±0.11 mg GAE/g DW. High levels (3.40±0.11 mg GAE/g DW), were found in extracts of samples, collected in the Site3. Moderate levels (2.93±0.03 mg GAE/g DW), were found in extracts of samples, collected in the Site2. Low levels (2.53±0.03 mg GAE/g DW), were found in extracts of samples, collected in the

Site1. The differences in total phenolic content, between *Quercus ilex* L.collection sites, were statistically significant ($P < 0.05$). In fact, the content of total phenols differs from one site to another.

This variation can be attributed to several factors, climatic and environment, geographical area, drought, diseases (Ebrahimi and*al.*,2008 ;Andarwulanand*al.*, 2010), to harvest time and stage of plant development (Miliauskas and*al.*,2004).In addition, genetic factors and growing conditions, may play an important role in the formation of secondary metabolites, including phenolic acids (Islam and*al.*,2003 ;Hashempourand*al.*, 2010). The method of extraction and quantification, also, influences the estimation of total phenols content (Lee and*al.*,2003 ;Viveket*al.*,2013).Phenols are very important plant constituents, because of their scavenging ability, on free radicals, due to their hydroxyl groups.Therefore, the phenolic content, of plants, may contribute directly to their antioxidant action and it is likely that, the activity of the extracts, is due to these compounds (Tosun and*al.*, 2009 ; Ghasemzadehand*al.*,2010 ; Dróżdż and Pyrzynska, 2018).This activity, is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, by donating a hydrogen atom or an electron, chelating metal ions, in aqueous solutions (Petti and Scully,2009), quenching singlet and triplet oxygen, or decomposing peroxides (Zheng and Wang,2001). Besides, the phenolic compounds, possess multiple biological properties, such as antitumor, antimutagenic and antibacterial properties, and these activities, might be, related to their antioxidant activity (Shui and Leong 2002 ;Hasmida and*al.*, 2015).

Total Flavonoid Content

The total flavonoid content, of *Quercus ilex*L. leaves, was shown in Table 4. The total phenolic content (mg/g), in methanolic extracts, expressed in quercetin equivalent (QE), varied between 1.83±0.02 and 2.72±0.01. The highest flavonoid concentration (2.72±0.01 mg QE/g DW), was registered in *Quercus ilex* L.leaves, extract from Site3, whereas the lowest amount of

flavonoid content (1.83±0.02 mg QE/g DW), were found in extracts from Site1. In fact, flavonoid contents, varied, significantly (*P<0.05*), between the sites.Amjad and Shafighi (2013),reported that annual and geographical climate differences, soil conditions and pesticide or herbicide use, may contribute to variations in antioxidant activity and to flavonoid content of plants. The concentration of flavonoids, in plant extracts, depends on the polarity of solvents, used in the extract preparation (Min and Chun-Zhao,2005). Flavonoids are class of secondary plant metabolites, with significant antioxidant and chelating properties. Beside phenolic compounds; the presence of flavonoids might, also, influence the antioxidant capacity. Antioxidant activity, of flavonoids, depends on the structure and substitution pattern of hydroxyl groups (Sharififar and*al.*,2008[16]. Flavonoids are polyphenolic compounds, with low molecular mass, found in leguminous, fruits, flowers, and leaves and they have several biological activities (Harbone and Williams, 2000). The polar extracts, of the leaves of *Quercus ilex*L., were abundant in flavonoids, in particular quercetin, isorhamnetin-3-O-glucopyranoside, flavonolacylatedglucosides and in the phenolic ellagic acid(Karioti and *al.*, 2009).Quantitative analysis, of the methanolic extract of the plant, revealed that it is a rich source of acylated flavonoid glucosides (Kariotiet *al.*,2010 ;Dróżdż and Pyrzynska, 2018).Flavonoids, as one of the most diverse and widespread group, of natural compounds, are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities, including radical scavenging properties (Agbor and*al.*,2005).

Table 4. Total Phenolic, Flavonoid Content and DPPH radical-scavenging activity (IC_{50}), from the leaves of *Quercus ilex* L., determined in three preserved state collection sites (Site 1), in moderately degraded state (Site 2) and in degraded state (Site 3), of National Park DjebelZaghouan

Sample site	Total phenolic content (mg GAE/g DW)	Total flavonoids content (mg QE/g DW)	IC_{50} (µg/mL)
Site1	2.53 ± 0.03^{c}	1.83 ± 0.02^{c}	102.66 ± 0.33^{c}
Site2	2.93 ± 0.03^{b}	2.24 ± 0.04^{b}	124.29 ± 0.45^{b}
Site3	3.40 ± 0.11^{a}	2.72 ± 0.01^{a}	134.83 ± 0.53^{a}
Positive reference (ascorbicacid)			48.31 ± 0.11^{d}

Values of the same column with different superscripts (a–c) are significantly different at $p < 0.05$.

DPPHradical-scavenging activity

A number of methods are available, for the determination of free radical scavenging activity, but the assay employing the DPPH, has received the maximum attention, owing to its ease of use and its convenience (Rout and*al.*, 2011).

As shown in Table 4 and Table 5, the DPPH free radical scavenging activity of *Quercusilex*L. sample extracts, was in concentration dependent manner. Indeed, the results obtained, indicated that *Quercus ilex* L. samples, collected from Site1 (19.35–80.13%),had, significantly, the highest inhibition percentages.The samples, collected from Site2 (17.35–78.13%), were, significantly, the second and those, collected from Site3 (15.25–74.28%), was the third (Table 5).

Table 5. The DPPH radical scavenging activities (%), of methanol extracts, of *Quercus ilex* L., determined in three preserved state collection sites (Site 1), in moderately degraded state (Site 2) and in degraded state (Site 3), of National Park Djebel Zaghouan

Sample site	Concentrations (µg/mL)			
	10	50	150	250
Site1	15.25^{d}	25.11^{d}	64.71^{d}	74.28^{d}
Site2	17.35^{c}	29.52^{c}	68.06^{c}	78.14^{c}
Site3	19.35^{b}	37.52^{b}	76.06^{a}	80.14^{b}
Ascorbic acid	39.97^{a}	57.61^{a}	71.83^{b}	81.37^{a}

Values of the same column with different superscripts (a–c) are significantly different at $p < 0.05$.

IC_{50}value, is negatively related to the antioxidant activity, as it expresses the amount of antioxidant, needed to decrease the radical concentration by 50% (Basuny and*al.*, 2012). The ability, of leaf extracts, to scavenge the DPPH radical, measured as IC_{50}, varied significantly from 102.66 to 134.83µg/mL. The samples, collected from Site3 showed high antioxidant activity, with their IC_{50} (134.83µg/mL). Moderate antioxidant activity, was found in leaves of Site2 (124.29µg/mL). Low levels, of antioxidant activity (102.66µg/mL), were found in leaves of Site1 (Table 4). The lower is the IC_{50} value, the higher is the antioxidant activity, of the tested

sample. Therefore, samples, collected form Site1, have maximum antioxidant activity, where, Site2 and Site3, have least antioxidant activity.

All Holm oak samples, showed free radical scavenging activity, but less than synthetic antioxidants (Table 5). The samples, collected from Site1, had the highest free radical scavenging, at all used concentrations, compared to others hom oak samples. It, may be, related to its contents from total polyphenol and flavonoid contents. In fact, various phytochemical components, especially polyphenols, are known to be responsible, for the free radical scavenging and antioxidant activities of plants (Atoui and*al.*,2005; Huang and*al.*, 2012). Phenolic compounds and flavonoids, have been reported to be associated with antioxidative action, in biological systems, acting as scavengers of singlet oxygen and free radicals (Asadujjaman and*al.*,2013). Total phenolics, generally, correlate with redox and antioxidant capacities, as measured by the DPPH method (Tabart and*al.*, 2006). Many studies, showed a correlation between antioxidant activity and phenolic content (Djeridane and *al.*,2006 ;Bhoyarand*al.*, 2011).

The DPPH scavenging activity, of the extract, is dependent on various biochemicals, besides, the polyphenolic contents (Agboret *al.*, 2005). Indeed, a study, performed by Babbaret *al.* (2011), showed that phenolic compounds, alone, are not fully responsible, for the antioxidant activity of plants. Other constituents, such as ascorbates, reducing carbohydrates, tocopherols, carotenoids, terpenes, and pigments, as well as, the synergistic effect among them, could, possibly, contribute to the total antioxidant activity.In addition, the chemical structure, of phenolics, play a role, in the free radical scavenging activity, mainly depending on the number and position of hydrogen donating hydroxyl groups, on the aromatic rings of the phenolic molecules (Bouayed and*al.*, 2011). Antioxidant activity, of plant extracts, also, depends on the type and polarity of the extracting solvent (Ismail and*al.*, 2004).

CONCLUSIONS

The results,found in this study, revealed that altitude and environmental conditions resulted, in significant changes, in both, leaf essential oil and fatty acid composition, in Holm oak (*Quercus ilex* L.). Nutritional potential [richness in MUFA, such as oleic acid (c9, C18:1), presence of essential fatty acids, such as linoleic acid (c9, 12-C18:2)], of the oil of Holm oak leaf, can provide opportunities for rational exploitation,in medicinal purposes and in the food industries.In addition, to its the wealth of essential oils and fatty acids, the leaves of *Quercus ilex*L., have a high content of phenolic compounds and a good antioxidant activity. Polyphenols are important natural antioxidants, play a major role in the prevention various pathological conditions. However, non-phenolic substances, can be responsible for the antioxidant activity of *Quercus ilex*L.. Therefore, further studies, are needed to identify which phenolic compounds, are responsible for the antioxidant activity of the species, and assess the way, in which the phenolic substances contribute to this activity.

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