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Olive Leaves, a Promising Byproduct of Olive Oil Industry: Assessment of Metabolic Profiles and Antioxidant Capacity as a Function of Cultivar and Seasonal Change

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Abstract: Olive (*Olea europaea* L.) leaves are an abundant byproduct in the olive oil industry characterized by high quantities of phenols. The content of these molecules in olive leaves may vary according to several factors, including cultivar, olive tree alternate bearing cycle, and seasonal variations. In the present study, leaves belonging to five Italian cultivars ('Biancolilla', 'Nocellara Etnea', 'Nocellara Messinese', 'Nocellara Siracusana', 'Zaituna'), cultivated in the same area, were collected from January to August and individually subjected to an appropriate extraction assay. Each extract was analyzed by means of HPLC/DAD/ESI-MS to determine its phytochemical profile and assess inter cultivar and seasonal variations. Moreover, total phenolics, ORAC, and DPPH assays were performed, in order to evaluate the Total Polyphenol Content and antioxidant potential. Seventeen different metabolites belonging to the subclasses of simple phenols, secoiridoids, hydroxycinnamic acids, and flavonoids were identified, quantified, and used as markers to monitor profile variations. The data obtained have shown that secoiridoids, particularly oleuropein, can be regarded as key molecules to discriminate among different cultivars and to assess variability in olive leaves during the flower and fruit development stage. All of the samples evidenced a remarkable antioxidant activity in terms of Radical Scavenging Activity, and as expected, some differences were observed among cultivars and depending on the harvest period through ORAC assay.

Keywords: *Olea europaea* L.; leaves extract; secoiridoids; flavonoids; hydroxycinnamic acids; seasonal variation

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1. Introduction

The olive tree (*Olea europaea* L.) is an evergreen drought and moderately salt-tolerant species that has been cultivated since ancient times for its oil and fruits in the Mediterranean area [1], of which is still one of the most iconic species owing to its ecological, economic, and cultural importance [2]. The olive tree has a high variability in fruit production, with fruit yields varying from 7–8 tons/ha to only a few hundred kilograms/ha depending on the local environment conditions [3]. Olive leaves are an abundant byproduct in the olive oil industry that amounts to roughly 10% of the total weight of the harvested olives and accumulate in high quantities during tree pruning [4]. Olive leaves contain high quantities of phenolic substances, thus representing a good source of added-value compounds with numerous health-promoting properties [5,6]. In fact, it has been demonstrated that olive leaf extracts possess high antioxidant and scavenging activities and could therefore be used in various applications [7–9]. Secoiridoids such as oleuropein,

dimethyloleuropein, oleuropein aglycone, ligstroside, ligstroside aglycone, and oleoside, together with simple phenols such as tyrosol and hydroxytyrosol, are typical of *O. europaea* leaves, which also contain flavonoid glycosides and flavonoids aglycones. Phenolic acids (ferulic, caffeic, chlorogenic, *p*-coumaric, homovanillic, and vanillic) were also found to be present in this matrix [10,11]. The content of these molecules in olive leaves may vary according to genetic and environmental factors, as often observed when dealing with plant secondary metabolites [12]. The cultivar seems to play a predominant role in differentiating the phenolic profiles of olive leaves [13,14], which are also affected by the olive tree's alternate bearing cycle [15] and by seasonal variations within the same cycle [14,16–18]. As part of our research activity, we proposed various applications for the improvement of nutritional value and shelf life of foods [19–22], also considering the employ of yeasts for biotechnological applications to obtain olive leaf extracts with enhanced bioactivity, where simple phenols, such as hydroxytyrosol, are retrieved in situ mainly via the control of oleuropein after hydrolysis [23,24] The purposes of this study were therefore (1) to screen a group of local olive cultivars according to the biological cycle of olive trees in search for the best cultivar in terms of oleuropein content, (2) to evaluate each cultivar for the optimal oleuropein accumulation period, and (3) to assess the antioxidant activities of each cultivar during seasonal change.

2. Materials and Methods

2.1. Chemicals and Reagents

All of the solvents and reagents used in this study: Folin–Ciocalteu phenol reagent, sodium carbonate (Na_2CO_3), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ethanol (≥96%), Trolox [(+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid],2,2-Azinobis (2-aminopropane), and hydrochloride (AAPH), were of analytical grade and purchased from Sigma–Aldrich (Milan, Italy). HPLC grade water, acetonitrile, and methanol were obtained from VWR (Milan, Italy). Luteolin, luteolin 7-*O*-glucoside, and apigenin 7-*O*-glucoside were provided by Extrasynthese (Lyon, France). Rutin (quercetin 3-*O*-rutinoside), apigenin, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, hydroxytyrosol, oleuropein, 3,4-dihydroxyphenilacetic acid (DOPAC), and fluorescein were obtained from Fluka (Sigma–Aldrich, Milan, Italy).

2.2. Olive Leaves Collection

The independent samples, reported in Table 1, (1 kg each) of leaves from the olive cultivars 'Biancolilla', 'Nocellara Etnea', 'Nocellara Messinese', 'Nocellara Siracusana', and 'Zaituna', coming from organic cultivations grown in the southeast of Sicily, Italy (37°04′09 N, 15°25′58 E), were collected from the same selected trees during eight months (January-August), comprising the vegetative phase (I, January–February), flowering (II, March–April), and fruit set/development (III, May–June and IV, July–August). The period September–December was not included in the study as the majority of the olive cultivars are in the fruit growth phase. During this very delicate period, the pruning process is not usually carried out. A list of the olive leaf samples of this study, together with their laboratory codes, is shown in Table 1.

Table 1. Coding of samples analyzed in this study.

Sample	Cultivar	Harvesting Period	Code
1	Biancolilla	January–February	**B-I**
2	Biancolilla	March–April	**B-II**
3	Biancolilla	May–June	**B-III**
4	Biancolilla	July–August	**B-IV**
5	Nocellara Etnea	January–February	**NE-I**
6	Nocellara Etnea	March–April	**NE-II**

7	Nocellara Etnea	May–June	**NE-III**
8	Nocellara Etnea	July–August	**NE-IV**
9	Nocellara Messinese	January–February	**NM-I**
10	Nocellara Messinese	March–April	**NM-II**
11	Nocellara Messinese	May–June	**NM-III**
12	Nocellara Messinese	July–August	**NM-IV**
13	Nocellara Siracusana	January–February	**NS-I**
14	Nocellara Siracusana	March–April	**NS-II**
15	Nocellara Siracusana	May–June	**NS-III**
16	Nocellara Siracusana	July–August	**NS-IV**
17	Zaituna	January–February	**Z-I**
18	Zaituna	March–April	**Z-II**
19	Zaituna	May–June	**Z-III**
20	Zaituna	July–August	**Z-IV**

2.3. Preparation of Olive Leaves Extracts (OLEs)

The plant material (Table 1) was oven-dried at 40 °C until constant weight using a drying oven (Electro-thermal Blast Drying Oven WLG-45B, Tianjin, China) and then milled through a grinder (AR1100, Moulinex, Paris, France). The dried leaves of each cultivar were individually milled using a grinder (AR1100, Moulinex, Paris, France).

The Olive Leaf Extracts (OLE) were prepared using distilled water as the extraction solvent, following the protocol previously reported [24]. Each OLE was then filtered using a 0.20 µm pore-size membrane filter (Millipore®, Burlington, MA, USA) and stored at −20 °C until use.

2.4. Qualitative and Quantitative Determination of Olive Leaves Extracts (OLEs) Polyphenols by HPLC-DAD and HPLC-ESI-MS

High-performance liquid chromatographic analyses were carried out on an Ultimate 3000 instrument equipped with a binary high-pressure pump, a Photodiode Array detector, a Thermostated Column Compartment, and an Automated Sample Injector. The collected data were processed through a Chromeleon Chromatography Information Management System v. 6.80 (Thermo Scientific, Milan, Italy). The OLE polyphenols were eluted, detected, and quantified according to Palmeri et al. [24]. HPLC-ESI-MS analyses on OLEs were performed using the same HPLC apparatus as described above, whilst ESI mass spectra were acquired by a Thermo Scientific Exactive Plu Orbitra MS (Thermo Fisher Scientific, Inc., Milan, Italy) using heated electrospray ionization (HESI II) interface. The data acquisition and analyses were performed using the Excalibur software (Thermo Scientific, Milan, Italy). The analyses were always carried out in triplicate.

2.5. Spectrophotometric Evaluation of Total Phenolic Content of Olive Leaves Extracts (OLEs)

The total phenolic content (TPC) of the OLEs was evaluated using the Folin–Ciocalteau (FC) method, as reported by Singleton et al. [25], with minor modifications. Briefly, 250 µL of each OLE sample was mixed with 1.25 mL of FC reagent and allowed to react for 3 min, then 2.5 mL of 20% sodium carbonate (Na_2CO_3) was added. The mixture was brought to a final volume of 25 mL with water and left to stand in the dark for 1 h at 25 °C. After that incubation period, the absorbance of each sample was recorded using a Perkin Elmer lambda 25 UV-Vis (PerkinElmer Italia Spa, Milano) setting at 725 nm. A calibration curve was created using gallic acid as standard, and the results were expressed as mg GAE (Gallic Acid Equivalent)/g dry leaf.

2.6. Antioxidant Activity of Olive Leaves Extracts (OLEs)

The Radical Scavenging Activity (RSA) was determined by using a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay following the protocol previously reported by Brand–Williams et al. [26] with minor modification. The assay was conducted by mixing 3 mL of DPPH solution (100 μM) with 50 μL of each OLE, containing 50 μL of methanol (instead of OLE), which was used as blank. Each reaction mixture was then incubated in the dark for 1 h at 25 °C. The absorbance was read at 515 nm using a Perkin Elmer lambda 25 UV-Vis spectrophotometer (PerkinElmer Italia Spa, Milano) against the blank.

The final RSA value was calculated as the average of the three replicates and expressed as a percentage by applying the following Equation (1):

$$\text{RSA \%} = [(\text{Absorbance blank} - \text{Absorbance sample})/\text{Absorbance blank}] \times 100 \quad (1)$$

The total reducing capacity was determined using the ORAC assay, as described by Scalzo et al. [27]. The measurements were performed on a Wallac 1420 Victor III 96-well plate reader (EG and Wallac, Turku, Finland) with fluorescence filters (excitation = 485 nm, emission = 535 nm). Fluorescein (116 nM) was used as a target for free radical attack by AAPH (153 mM), used as a peroxy radical generator. The reactions were performed at 37 °C, pH 7.0, with Trolox (1 μM) as a control standard and phosphate buffer as a blank. All of the solutions were freshly prepared prior to analysis. All of the samples were diluted with phosphate buffer (1:25–100, *v*/*v*), and the results were expressed as millimoles of Trolox Equivalent (mmol TE) on grams of dry leaves.

2.7. Statistical Analysis

The data obtained from the Folin–Chocalteau assay were analyzed separately by using the Statistical package software Minitab™ version 20.(Commonwealth of Pennsylvania, USA) The differences between experimental groups were determined on mean values with a one-way analysis of variance (ANOVA), and the significant ($p < 0.05$) differences (mean separation) between the treatments were carried out by Fisher's least-significant difference test.

3. Results and Discussion

3.1. HPLC-DAD and HPLC-ESI-MS

The HPLC-DAD chromatograms corresponding to the five cultivars of this study during the first observation period are depicted in Figure S1 (see Supplementary Material). Seventeen peaks were tentatively identified by comparing their relative retention times and spectral data (UV-Vis and MS) with those of corresponding analytical standards when available; the assignments were further corroborated by the literature (Table S1, Supplementary Material). All of the compounds identified belong to the class of polyphenols and can be further divided into different subgroups according to their chemical structure: the simple phenols hydroxytyrosol, its glucoside, and DOPAC (dihydroxyphenyl acetic acid) characterize the first part of the chromatogram (from 5 to 7 min) in all of the cultivars analyzed; the portions ranging from 9 to 13 min are instead clearly dominated by hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic acid,; see also Table S1) and their derivatives (chlorogenic acid, and verbascoside). The remaining part of the chromatograms, from 9 to 20 min, is characterized by the presence of flavonoids (rutin, luteolin 7-O-glucoside, apigenin exoside, apigenin 7-O-glucoside, luteolin, and apigenin, see Table 1) and secoiridoids (oleuropein, oleuropein aglycone, and ligstroside). Secondary metabolites bearing a simple phenol, such as tyrosol and hydroxytyrosol, or more complex structures such as secoiridoids oleuropein and ligstroside are regarded as peculiar for olives and olive products [4,28]. Once identified and quantified, all of the 17 compounds were used as markers to highlight the differences and similarities among the cultivars and monitor profile changes during the time intervals considered. The quantitative data are reported in Table 2. When focusing purely and simply on a qualitative point of view, the

portion of the chromatograms (Figure S1 in Supporting Material) more variable within cultivars is that from 11 to 16 min, which includes hydroxycinnamic acids, flavonoids, and secoiridoids (verbascoside, *p*-coumaric acid, ferulic acid, rutin, luteolin 7-O-glucoside, apigenin hexoside, oleuropein, apigenin 7-O-glucoside, also see Table S1). Regarding the quantitative data, all of the cultivars showed elevated phenolic contents during the spring period (II and III), with the highest values observed in 'Nocellara Siracusana' (33.07 ± 0.55 mg/g) during July-August (Table 2). These data fall in line with the study of Benincasa et al. [18], which assesses how ten Italian cultivars present a high content of oleuropein in the same period. This trend was observed for all of the cultivars considered except 'Zaituna', whose metabolite accumulation instead starts in period II and peaks in period III, being staggered with respect to the others (Table 2). This phenomenon is definitely genetic, and it is probably related to the different flowering periods of this cultivar. The results here obtained agree with those reported by Heimler et al. [29] for flavonoids and with the work of Sahin and others [30], who registered a general drop in the olive leaves's polyphenols (especially oleuropein) from spring to summer within a collection of 20 olive cultivars from Texas. The same fall in polyphenol content during summer (August) was observed by Talhaoui et al. [31] while studying the seasonal changes in olive leaves from six cultivars in Andalusia, although the authors considered a different time interval (fruit set to fruit ripening). More recently, Blasi and coworkers [14] evaluated the phenolic composition of olive leaves from four different cultivars collected in four different periods (December-September). Their results match with those reported here, as the authors observed significant differences in oleuropein content among the cultivars and found in December and March the highest contents of bioactive compounds. Oleuropein is the most discriminant metabolite according to our results (Table 2), as better displayed in Figure 1. From this picture, it is possible to note that all cultivars evidenced the highest content of oleuropein in the II period, with again the sole exception being the cultivar "Zaituna".

Table 2. Content of individual metabolites in the olive leaves extracts (OLEs) from five cultivars and four observation periods object of this study. See also Figure S1, Table S1 and text for further details.

		Metabolite Content, mg/g Dry Vegetable Material [a]																			
		OLE [b]																			
Pea	**Compound**	**B-I**	**B-II**	**B-**	**B-**	**NE-I**	**NE-II**	**NE-**	**NE-**	**NM-I**	**NM-**	**NM**	**NM**	**NS-**	**NS-II**	**NS-**	**NS-**	**Z-I**	**Z-II**	**Z-III**	**Z-**
1	hydroxytirosol glucoside	0.18	2.88	0.19	0.22	0.40	0.538	0.70	0.22	0.50	1.18	0.12	0.20	0.11	2.219	0.68	0.04	0.17	0.20	0.925	0.24
2	hydroxytirosol	0.26	0.40	0.28	0.14	0.43	0.63	0.23	0.18	0.37	0.13	0.19	0.18	0.14	0.81	0.21	0.13	0.60	0.84	0.349	0.20
3	dihydroxyphenylacetic	0.13	0.30	0.09	0.11	0.12	0.09	0.12	0.16	0.08	0.17	0.22	0.09	0.11	0.16	0.10	0.07	0.12	0.10	0.187	0.12
4	chlorogenic acid	0.16	0.34	0.11	0.09	0.14	0.22	0.04	0.10	0.26	0.17	0.05	0.05	0.11	0.32	0.05	0.05	0.12	0.19	0.144	0.11
5	caffeic acid	0.00	0.02	0.00	0.00	0.038	0.038	0.004	0.006	0.02	0.048	0.00	0.00	0.00	0.03	0.006	n.d.	0.01	0.01	0.004	0.00
6	verbascoside	0.13	0.54	0.04	0.04	1.23	1.79	0.26	0.15	1.41	0.99	0.09	0.04	0.08	1.808	0.33	0.175	0.24	0.26	0.184	0.06
7	*p*-coumaric acid	0.05	0.07	0.07	0.03	0.09	0.095	n.d. [c]	n.d. [c]	0.148	0.081	0.02	0.05	0.03	0.078	0.042	0.052	n.d. [c]	n.d [c]	n.d. [c]	n.d. [c]
8	ferulic acid	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	n.d [c]	n.d. [c]	n.d. [c]	n.d. [c]	0.10	0.14	0.106	0.04
9	rutin	0.11	0.18	0.10	0.06	0.31	0.32	0.22	0.34	0.31	0.37	0.06	0.08	0.10	0.53	0.28	0.20	0.18	0.39	0.407	0.24
10	luteolin 7-*O*-glucoside	0.79	1.51	0.56	1.22	2.86	3.23	2.77	3.23	2.58	2.23	1.12	1.13	3.01	4.88	2.92	1.63	0.76	1.13	1.554	0.79
11	apigenin hexoside	0.31	0.32	0.33	0.23	0.28	0.31	0.31	0.42	0.41	0.30	0.29	0.36	0.25	0.45	0.33	0.15	0.29	0.28	0.332	0.22
12	oleuropein	4.35	33.34	3.39	4.36	16.28	24.35	19.17	18.17	16.81	32.05	5.62	5.81	6.16	33.15	21.41	13.50	6.31	4.62	16.62	5.55
13	apigenin 7-*O*-glucoside	0.36	0.59	0.18	0.29	1.19	1.27	0.83	1.46	1.05	0.90	0.43	0.40	1.02	1.99	1.07	0.66	0.30	0.53	0.509	0.30
14	ligstroside	0.53	4.13	0.28	0.49	1.97	3.20	1.14	1.30	2.53	2.88	0.51	0.47	0.21	3.06	1.907	0.908	0.66	0.72	0.898	0.43
15	oleuropein aglicone	0.12	0.378	0.18	0.16	0.54	0.54	0.76	0.46	0.54	0.53	0.25	0.36	0.29	0.73	0.63	0.38	0.13	0.19	0.356	0.17
16	luteolin	0.02	0.02	0.00	0.01	0.069	0.10	0.02	0.07	0.11	0.05	0.03	0.01	0.05	0.12	0.06	0.04	0.05	0.11	0.045	0.02
17	apigenin	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	0.019	0.01	n.d. [c]	0.01	n.d. [c]	n.d. [c]	n.d. [c]	n.d. [c]	0.00	n.d. [c]	n.d. [c]	0.01	n.d [c]	n.d [c]	n.d. [c]	n.d. [c]

[a] as mean of three replicates; [b] see material and methods; [c] not detected

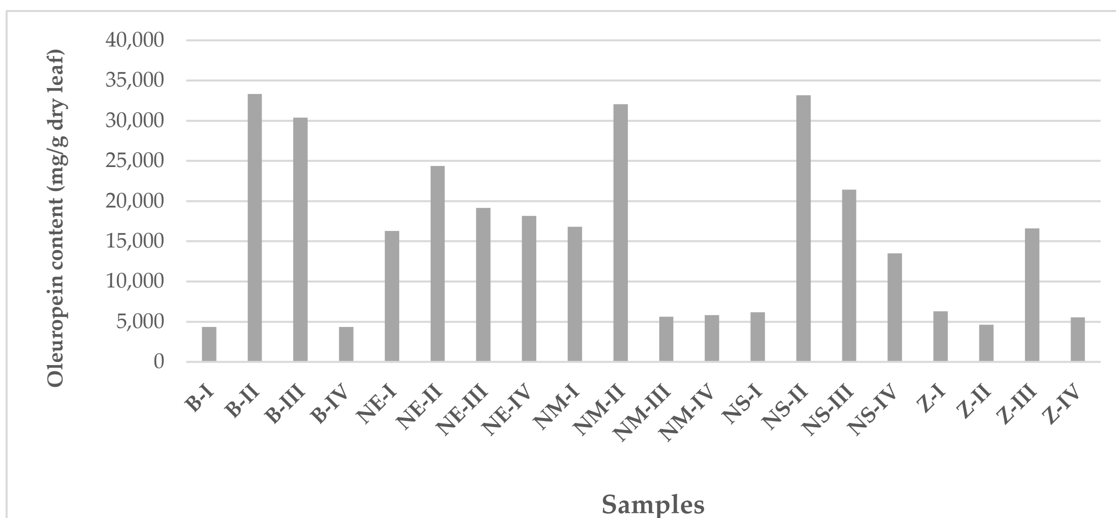

Figure 1. Oleuropein content of samples under study (Table 1) expressed as mg/g dry leaf and evaluated using high performance liquid chromatographic technique.

3.2. Spectrophotometric Evaluation of Total Polyphenols Content and Antioxidant Activity of Olive Leaves Extracts (OLEs)

Figure 2 displays the total polyphenol content of the OLEs evaluated in the present study expressed as mg GAE/g dry leaf. The data obtained from the Folin–Ciocalteau assay (Figure 2) show that all of the samples present a good amount of TP, although, for each cultivar, it is possible to observe a different trend strictly correlated with the harvesting season. In particular, during the I period (January–February), the samples of NE-I and NM-I evidenced significantly ($p < 0.05$) higher values, respectively, of 26.4 ± 2.6 and 24.0 ± 1.5 mg GAE/g dry leaf, while the lowest polyphenols content were observed in the B-I and NS-I samples, which, respectively, registered the values of 16.3 ± 0.5 and 16.5 ± 0.2 mg GAE/g dry leaf (Figure 2a). In the II period (March–April), the samples of B-II, NE-II, and NM-II evidenced significantly ($p < 0.05$) higher values of total polyphenols, ranging from 30.1 ± 1.4 (sample B-II) to 29.1 ± 0.25 mg GAE/g dry leaf, while the lowest value was observed in the samples of Z-II, which registered a polyphenols content equal to 22.5 ± 1.1 mg GAE/g dry leaf. These results could be attributed to the oleuropein content that, as already reported in Section 3.1, was very high in the II period (March–April) for all of the samples with the exception of the cultivar "Zaituna" (Figure 2b). During the III period, the NE-III, NS-III, and Z-III samples evidenced significantly ($p < 0.05$) higher TP contents (Figure 2c), with the values of 29.3 ± 2.2, 28.9 ± 2.9, and 30.5 ± 2.0 mg GAE/g dry leaf. In the last harvest period (July–August) the only sample that still had an elevated content of TP was NS-IV (Figure 2d). Despite the significant differences observed among the cultivars, our TP data are partially in accordance with those reported by Heimler et al. [29] and Benincasa and coworkers [18], whom both evidenced an increase in TP content during the period from spring to summer.

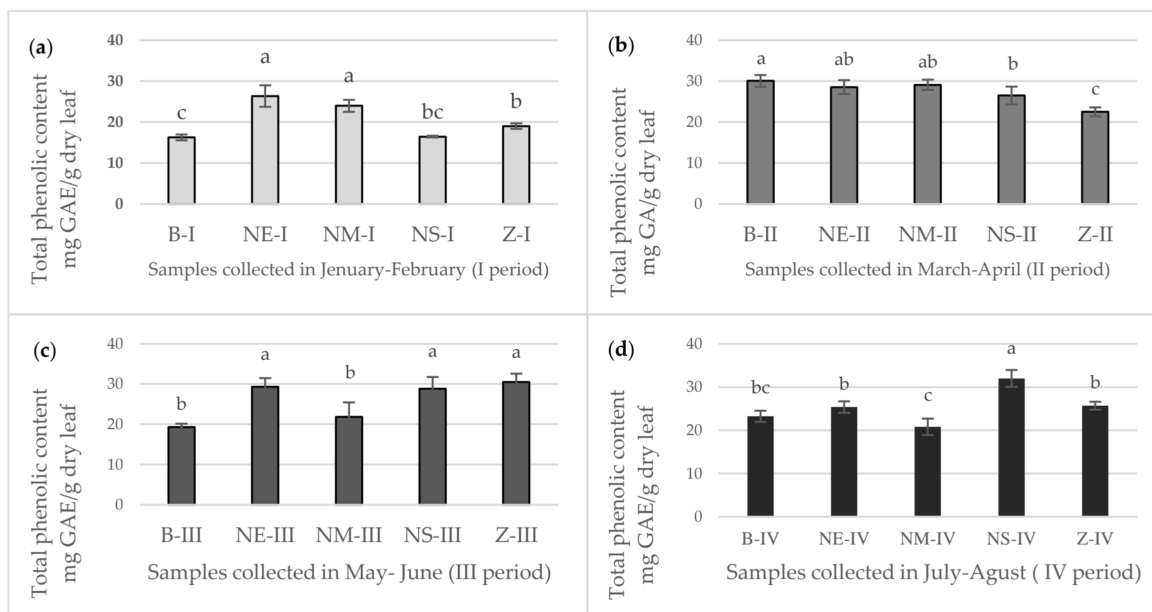

Figure 2. Spectrophotometric evaluation of total polyphenols content (TPC) of samples under study (Table 1) expressed as mg GAE/g dry leaf. (**a**) Samples collected in January–February (I period); (**b**) Samples collected in March–April (II period); (**c**) Samples collected in May–June (III period); (**d**) Samples collected in July–August (IV period). Vertical bars indicate the standard deviation of the mean. Different letters over the bars within each harvest period (I, II, III, or IV) are significantly different according to Fisher's least significant difference test ($p < 0.05$).

Regarding antioxidant activity, Figure 3 displays the results obtained for all of the samples under study (see Table 1 for sample list). The DPPH assay evidenced that a high RSA% (>80%) characterizes all of the samples (Figure 3a). The ORAC method (Figure 3b) is widely used to estimate the antioxidant capacity of the most consumed foods and beverages [32]. Boss et al. [33] reported a collected study to compare the polyphenol content of OLEs with respect to extra virgin olive oil (EVOO) and its anti-cancer effect and health benefits. Therefore, of great importance is the knowledge of the evolution of these compounds in terms of antioxidant activity during the growth of olive trees. The results evidence that almost all of the cultivars present the highest values during periods II–III, corresponding to the peak of metabolite accumulation (Table 1).

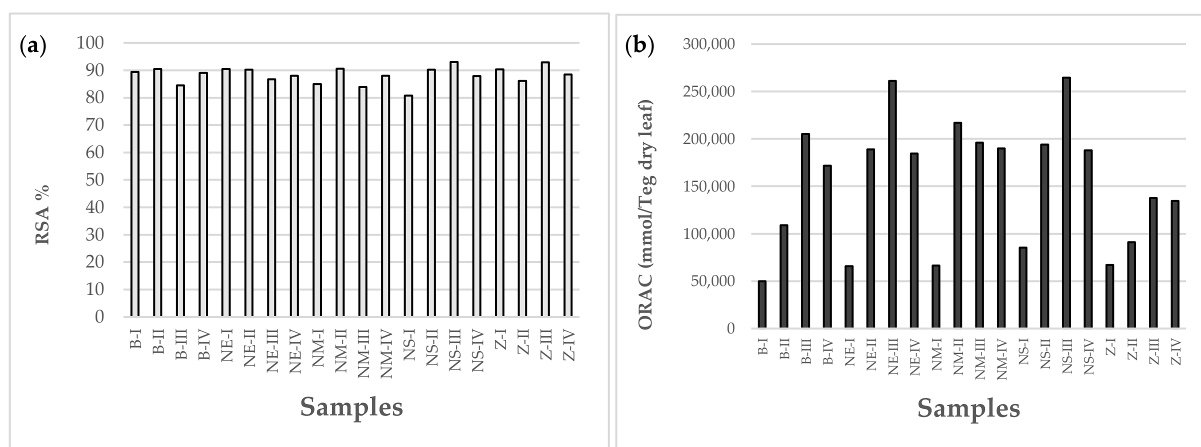

Figure 3. Assessment of antioxidant activity through (**a**) DPPH and (**b**) ORAC assay.

The increase in antioxidant activity is probably related to the olive leaves antioxidant defense mechanism [18], suggesting that the higher antioxidant activity observed is not directly correlated with oleuropein and TP content.

4. Conclusions

OLEs from five Italian cultivars 'Biancolilla', 'Nocellara Etnea', 'Nocellara Messinese', 'Nocellara Siracusana', and 'Zaituna' were analyzed for their polyphenol profiles and content in the search for inter-cultivar differences/similarities and in order to monitor metabolite accumulation across eight months, from the vegetative phase to the fruit development. The antioxidant activities (DPPH and ORAC tests) were also measured for the same extracts. The ORAC assay allows for the discrimination of the antioxidant activity between the different cultivars and harvesting periods. The data obtained show that there are substantial inter-cultivar differences during the first period, which is the period of dormancy of the olive trees. During the vegetative growth corresponding to periods II–IV, a different concentration in bioactive compounds was observed. The trend is similar in all of the cultivars examined. These data confirm that the study of metabolic profiles is a useful tool to discriminate among cultivars and support genetic control over the environment in the OLE metabolic accumulation process. The results obtained allow for the selection of the best cultivar and the best collection period to obtain extracts that are rich in oleuropein.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092007/s1, Figure S1: HPLC-DAD profile, visualized at 280 nm of the olive leaves extracts (OLEs) object of this study during the first observation period (January–February). Numbers refer to Table S1 and text. B = 'Biancolilla'; NE = 'Nocellara Etnea'; NM = 'Nocellara Messinese'; NS = 'Nocellara Siracusana'; Z = 'Zaituna'. See text for further details; Table S1: Phenols identified in OLEs from five Sicilian cultivars object of this study using HPLC/DAD and HPLC/ESI-MS. See Figure S1 for numbering and text for details.

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