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Quantification of Total Phenols, Tannins, Anthocyanins Content in *Myrtus communis* L. and Antioxidant Activity Evaluation in Function of Plant Development Stages and Altitude of Origin Site

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Abstract: Most of the biological properties of myrtle (*Myrtus communis* L.) are linked to the antioxidant activity of the phenolic compounds present in the extracts. In this study, the content of total phenols, tannins, and anthocyanins of acidified ethanol extracts of berries and leaves of five myrtle cultivars was quantified during the whole fruit development. In some development stages, the antioxidant activity (AA) of both tissues was evaluated with FRAP, ABTS, DPPH, β-carotene assays, and spectroscopic method (EPR). The trend of AA in leaf and berry extracts was different among genotypes and evaluation assays. In general, leaves showed with DPPH assay, the highest AA during autumn season, while with FRAP and β-carotene was higher at 120 DAF (days after flowering) corresponding to summer season. In berries, the AA with DPPH, ABTS, and EPR assays was the highest for all cultivars at 150 DAF and 210 DAF, while with β-carotene assay was higher in the first development stage. Both in leaf and berry, the total phenols and tannins contents influence the AA depending on the assay used, while the berries anthocyanins seem to play a minor role. The altitude of the site of origin differently affected the AA of myrtle leaf and berry. The study showed the potentiality to use the studied myrtle genotypes for further evaluation on biological applications of antioxidant activity.

Keywords: myrtle berry; leaf; tissue extract; phenolic compounds; Electron Paramagnetic Resonance

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1. Introduction

Myrtle (*Myrtus communis* L.), belonging to the Myrtaceae family, is present on the coasts of Portugal, southern France, Spain, southern Turkey, southern Italy, and other areas like Greece, Israel, Lebanon, Iran, Libya, Morocco, Algeria, and Tunisia. In Sardinia (Italy) myrtle grows spontaneously in neutral or sub-acidic soils and areas below 800 m of altitude [1]. Traditionally, Mediterranean folk medicine uses this plant for its carminative and astringent functions, as an antifertility agent, to treat respiratory diseases, for intestinal inflammation and menstrual problems [2]. This medicinal and aromatic plant received great attention in the last years for the large appreciation of the liqueurs produced by its berries (red liqueur) and leaves (white liqueur). The myrtle industry encouraged a domestication process of the species to integrate the harvests of biomasses from wild plants with those from selected cultivars with known chemical composition and quality attributes [1]. The availability of myrtle cultivars may be interesting for nutraceutical and medicinal industries.

Plant extracts, including those from myrtle berries and leaves, attract consumers and researchers for the high concentration of phenolic compounds involved in the scavenging of ROS (reactive oxygen species) and RNS (reactive nitrogen species) [3,4] which have been associated to Parkinson's [5], Alzheimer's [6], cardiovascular diseases [7], and diabetes [8]. Moreover, and as other foods rich in natural antioxidants, they are the object of numerous research [9–13].

The antioxidant properties of food are associated to the action of single bioactive compounds, to their interactions and to the interactions among antioxidants and other components of the food matrix [14]. Moreover, the type of chemical extraction largely affected the evaluation of both total antioxidant activity of the matrices, and of the role of the extractable and non-extractable biomasses fractions [14,15].

Myrtle leaves are rich in phenolic acids (gallic, caffeic, and ellagic acids), flavonol derivatives (myricetin, quercetin, and kaempferol derivatives), and ellagitannins (such as galloyl hexose derivatives) [16]. Berries contain anthocyanins (principally in berry skin and, partially, in the pulp) mainly delphinidin3-O-glucoside, petunidin3-O-glucoside, and malvidin 3-O-glucoside [17], flavonols, flavonols glycosides, phenolic acids, and flavanols [18]. Plant genotype, growing site, climatic conditions, maturation stage, tissue nature control the quantitative and qualitative composition of phenolic compounds and their antioxidant activity [19].

In previous studies, the antioxidant activity of myrtle, red and white myrtle liqueurs, and other derived myrtle products, was measured with ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid), DPPH (1,1-diphenyl-2-picrylhydrazyl), FRAP (ferric reducing antioxidant power), LDL (low-density lipoproteins) oxidation, β-carotene-bleaching methods, and the spin trapping method coupled with EPR (Electron Paramagnetic Resonance) [20–23].

These methods, based on different antioxidant mode of action, highlight the high antioxidant activity of myrtle plant tissues and its derivate food products [24–26].

Moreover, it is worth noticing that most of the biological properties of myrtle extracts are associated to their antioxidant activity [27,28]. Some of these demonstrated biological properties include neuro-protective effects [29], antimutagenic [30], and anti-hepatic ischemia [31].

According to Cruciani et al., [4] myrtle extracts controlled the ageing process in cells exposed to oxidative stress, suggesting their use in the prevention of senescence-associated diseases. Similarly, the extracts obtained from liqueur industry by-products counteract cells stress conditions and control cell senescence [28]. These studies suggest the use of myrtle extracts in nutraceutical formulations.

For this reason, it is essential to identify the best harvest time to maximize the antioxidant activity of myrtle berries and leaves [32]. Indeed, antioxidant activity and its development may vary among genotypes according to the leaf age, as well as to the polymorphism of myrtle fruit, i.e., the presence of pigmented and unpigmented genotypes. The unpigmented berry, although lacking anthocyanins, has an antioxidant power equal to that of the pigmented berry, due to the high content of gallic and ellagic acids [24].

The main objective of this study was to evaluate the influence of tissue developmental stages on the antioxidant activity of myrtle berry and leaf ethanol extracts, using different genotypes and assays in vitro. Furthermore, total phenolic compounds, total tannins, and anthocyanins accumulation in the plant, as well as the altitude of the site of origin of the genotypes were correlated to the antioxidant activities. Five myrtle cultivars were analysed, one with unpigmented fruits, to assess differences in antioxidant activity according to fruit polymorphism. Cultivars analysed were selected during the myrtle domestication program [1], and the obtained data should be considered in the light of a further breeding objective for the selection of plus variant cultivars with high antioxidant value to destine to pharmacological and food uses.

2. Materials and Methods

2.1. Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid), ABTS (2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), quinolinic acid (pyridine-2,3- dicarboxylic acid), Iron (II) sulphate hexahydrate and Iron (III) chloride hexahydrate were purchased from Merck (Darmastadt, Germany) Sigma. DMPO (5,5 dimethyl-1-pyrroline *N*-oxide) was purchased from Enzo Life Sciences (Milan, Italy). All other reagents and standards used in this study were purchased from Carlo Erba reagents S.r.l (Milan, Italy).

2.2. Plant Material Sampling and Extracts Preparation

The leaves and the berries of five myrtle cultivars ('Giovanna', 'Grazia', 'Maria Antonietta', 'Maria Rita', 'Sofia') were used to prepare the extracts. One of the cultivars ('Grazia') had white berries while the others had pigmented fruit. Myrtle plants were grown at the experimental orchard located at the "Antonio Milella" station of the University of Sassari (Central Western Sardinia 39°54′12″ N, 8°37′19″ E), but were originally collected in Sardinian sites at different altitude as reported in Table 1.

Table 1. Analysed five cultivars and its sites of origin. The altitudes and coordinate of the in sites of origin are reported.

Cultivar	Site of Origin	Altitude (m Above Sea Level)
'Giovanna', 'Grazia'	Rumanedda (40°33′36″ N 8°18′54″ E)	164
'Maria Antonietta'	Cuglieri (40°11′20″ N 8°34′02″ E)	248
'Sofia'	Laconi (39°51′09.71″ N 9°03′07.37″ E)	637
'Maria Rita'	Capoterra (39°10′30.67″ N 8°58′15.07″ E)	54

Table 2 reports some agrometeorological parameters of the meteorological station closest to the experimental field recorded during the harvest years.

Table 2. Meteorological parameters recorded in the experimental field (Fenosu site) during 2019. T = air temperature; RH = relativity humidity.

Meteorological Records	Months											
	J	F	M	A	M	J	J	A	S	O	N	D
T max (°C)	17.1	18.8	21.6	24.8	26.8	39.2	37.2	38.3	34.2	30.9	23.1	21.1
T min (°C)	0.0	0.5	4.1	3.7	5.8	12.3	17.2	15.9	14.2	11.4	5.9	2.5
T mean (°C)	8.4	9.5	12.7	14.1	16.0	24.1	26.1	26.0	23.0	19.6	14.2	13.1
Rainfall (mm)	0.0	91.0	16.2	16.6	58.4	55.4	1.8	2.4	17.2	34.8	46.2	290.4
RH max (%)	100.0	92.0	92.0	92.0	93.0	91.0	92.0	92.0	93.0	92.0	93.0	93.0
RH min (%)	43.0	22.0	30.0	33.0	30.0	24.0	28.0	25.0	28.0	39.0	55.0	42.0
RH mean (%)	82.0	75.0	71.0	73.0	73.0	64.0	67.0	68.0	72.0	74.0	82.0	79.0

The samplings were carried out during eight stages of development, at 0, 30, 60, 90, 120, 150, 180, 210, 225 days after flowering (DAF) that correspond to the sampling of June, July, August, September, October, November, early December, and late December of 2019. The berries and leaves sampling were done randomly on a plot of 20 plants for each cultivar. A graphical representation of sampling times of samples is shown in Figure 1. The first and last plant of the plot were excluded by the sampling as border in the row, and the remaining 18 plants were arranged in three subplots of six plants each, which represented the three biological replications. All the 18 plants were sampled at each time.

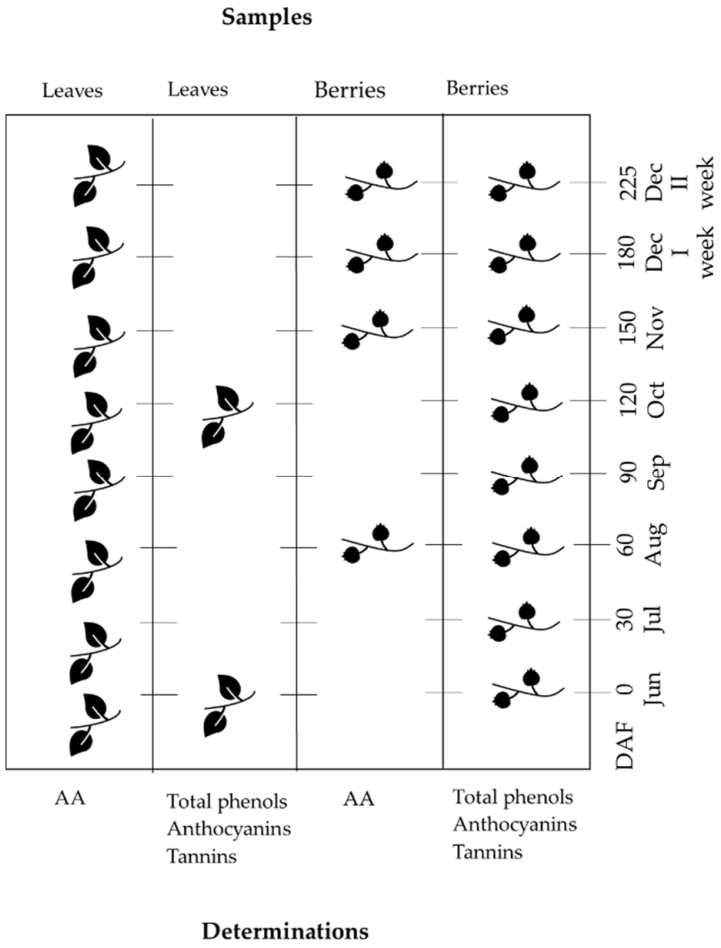

Figure 1. Graphical representation of the timing of sampling of myrtle berries and leaves.

Acidified ethanol was used as extraction solvent. Ethanol is one of the most employed solvents for the extraction of phenolic compounds; moreover, a hydroalcoholic extract is the base for myrtle liqueur production [33–35]. Acidification allows avoiding oxidation and increases extraction of compounds.

The berries (peel and 1 mm of pulp) and leaves were ground with liquid nitrogen. Ground tissue (0.6 g) was extracted with 15 mL of acidified ethanol (0.1% HCl) for 12 h at room temperature in the dark. Afterwards, the infusion was filtered with filter paper (Whatman No. 4) and stored at −20 °C before analysis. Each extract has been replicated three times. For each tissue sample, the dry matter was determined, after drying of 1 g of sample at 100 °C for 24 h.

For the evaluation of antioxidant activity, the analyses were carried out at selected stages: for berries extracts at 60, 150, 210, and 225 DAF; 0 and 120 DAF for leaves extracts. Leaf's sampling dates were chosen according to the completed development stage of the spring shoots (0 DAF) and autumnal maturity of the same shoots (120 DAF). Each chemical determination was carried out in triplicate.

2.3. Total Phenols, Anthocyanins, and Tannins Content

The determination of total phenols was carried out with the colorimetric method of Folin–Ciocalteu [36]. An aliquot (0.5 mL) of diluted ethanol extract was mixed with deionized water (35 mL) and 2.5 mL of Folin–Ciocalteu reagent; after 3 min of incubation, a sodium carbonate solution (20% in water) (5 mL) was added. The solution was incubated at 70 °C for 20 min and was brought to volume (50 mL) with deionized water. Absorbance was read at 750 nm with UV–VIS spectrophotometer CARY 50 VARIAN (Amsterdam,

The Netherlands). The results were expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g DW). For calibration curve, gallic acid concentrations were used between 0.05–0.5 mg/mL (R^2 = 0.99).

The total anthocyanin content was determined following the method described by Lee et al. [37]; the diluted extract was mixed with pH 1 buffer solution (potassium chloride, 0.025 M). The blank was prepared by adding to the same extract a pH 4.5 buffer solution (sodium acetate, 0.4 M). After 30 min of incubation at room temperature, the absorbance was read at 520 and 700 nm against a blank. For concentrations determination, the equation reported by Lee et al. was used [37]. Results were expressed in mg of cyanidin 3-glucoside equivalent (C3G)/g DW.

Tannins were determined according to Fadda and Mulas [38]. Four mL of the diluted extract was mixed with 2 mL of ethanol and 4 mL of the vanillin solution (1% vanillin in 70% sulphuric acid). After 30 min of incubation at room temperature, the absorbance was read at 500 nm. The results were expressed as mg of catechin equivalent (CE)/g DW based on the calibration curve (0.05–0.5 mg/mL of catechin, R^2 = 0.99).

2.4. Antioxidant Activity (AA) Assays

2.4.1. ABTS Assay

The method described by Re et al. [39] with some modifications was used to determine ABTS radical scavenging activity. The ABTS+ cation radical was produced by mixing ABTS stock solution (7 mM in water) and 2.45 mm potassium persulfate in a ratio of 1:0.5 respectively and was stored in the dark at room temperature for at least 16 h before use. The ABTS radical was diluted with water until the absorbance measured at 734 nm reached the value of about 0.7. The ABTS solution was prepared fresh before each analysis.

Fruit extract (5 µL) and leaf extract (2.5 µL) was mixed with 1000 µL of ABTS diluted. The reaction mix was incubated for 6 min in the dark at room temperature and the absorbance was immediately read at 734 nm using CARY 50 Scan Uv143 Vis VARIAN spectrophotometer (Amsterdam, The Netherlands). The calibration curve was prepared using a range of 0–4 mM (R^2 = 0.98) of Trolox reagent. Results were expressed as mmol of Trolox equivalent/g dry weight (mM TE/g DW).

2.4.2. FRAP Assay

The FRAP method has been carried out according to Benzie and Strain [40]. The FRAP reagent was prepared with 2.5 mL of 10 mM TPTZ, 2.5 mL of 20 mM $FeCl_3$- $6H_2O$ and 25 mL of 0.3 mM acetate buffer pH 3.6. The FRAP reagent was prepared fresh each time and warmed at 37 °C. A volume of 5 µL of berries or leaves ethanolic extract or ethanol (for the blank) was mixed with 900 µL of FRAP reagent and 115 µL of distilled water. The mix was incubated at 37 °C for 30 min and the absorbance was read at 595 nm using Cary 50 spectrophotometer. The calibration curve was prepared whit Trolox reagent at different concentrations (0.1, 0.4, 1, 2, 3 mM; R^2 = 0.99). The results were expressed in mmol of Trolox equivalent/g dry weight (mM TE/g DW).

2.4.3. DPPH Assay

The DPPH assay was carried out following the procedure reported by Sanna et al. [22], with some modifications. Briefly, a volume of 100 µL of ethanol acidified extract, properly diluted, was mixed with 100 µL of DPPH 1 mM solution (prepared in ethanol) and 1800 µL of ethanol. The mix was incubated at room temperature in the dark for 30 min, and then, the absorbance was read using Cary 50 spectrophotometer at 517 nm. The blank was prepared with 100 µL of DPPH solution and 1900 µL of ethanol. Trolox was used as a standard for calibration curve (range between 6–21 µM; R^2 = 0.97). The results were expressed in micromole of Trolox equivalent/g of dry weight (µmol TE/g DW).

2.4.4. β-Carotene Bleaching Assay

To provide a comprehensive view of the antioxidant potential of myrtle berries and leaves, the peroxyl radical (LOO•)-inhibiting capacity of the extracts was evaluated. Consequently, the β-carotene bleaching assay was performed following the method described by Aidi Wannes et al. [20] with some modifications. This assay was chosen to evaluate Two mg of β-carotene were dissolved in chloroform; one aliquot of this solution (4 mL) was mixed with 40 mg of linoleic acid and 400 mg of Tween 40. The chloroform in the solution was evaporated under a rotary vacuum at 50 °C, and then 100 mL of deionized water was added and mixed well. Three mL of this emulsion were added to 0.2 mL of sample extract. The control contained 0.2 mL of ethanol 3 mL of emulsion. BHT was used for comparison. The absorbance was immediately read (t = 0) at 470 nm and, a second time, after incubation in a water bath at 50 °C for 120 min (t = 120).

The antioxidant activity was expressed as antioxidant activity coefficient (CAA) calculated according to the equation reported by Klančnik et al. [41]:

$$CAA = 1 - (As(t = 0) - As(t = 120)/Ac(t = 0) - Ac(t = 120))$$

As = Absorbance of sample extract
Ac = Absorbance of control

2.4.5. Spin Trapping Assay of the •OH Radical

The •OH radical scavenging activity was determined with the spin trapping method coupled with Electron Paramagnetic Resonance Spectroscopy according to Fadda et al. [21].

The Fenton reaction was used to produce hydroxyl radicals using, as Fe (II) source a Fe (II) quinolic acid complex, prepared by mixing an aqueous $FeSO_4$ solution with quinolinic acid in order to have a final concentration of 0.1 mM and a ligand to metal ratio of 5:1. DMPO (261 µM) was used to entrap the hydroxyl radicals produced by the reaction of Fe (II) quinolinic acid complex with hydrogen peroxide (0.03%). Ten µL of the extracts properly diluted were used to evaluate the hydroxyl radicals scavenging activity.

The experiments were carried out at room temperature with a Bruker EMX spectrometer operating at the X-band (9.4 GHz) and a Bruker Aqua X capillary cell. EPR spectra were recorded immediately after the preparation of the reaction mixture; the concentration of the DMPO-OH adduct was estimated by the double integration of spectra. Results were expressed as µmol of gallic acid equivalents (mmols GAE/g DW) based on a calibration curve (gallic acid 80–200 mM, $R^2 = 0.99$). Three replications were performed for each extract. The EPR instrument was set under the following conditions: modulation frequency 100 kHz, modulation amplitude 1 G, receiver gain 1×10^5, microwave power 20 mW.

2.4.6. Data Treatment and Statistical Analysis

Results of phenolic compounds determination and AA are expressed as the means and standard deviations of three biological replicates. The results of the phenolic compounds were submitted to a one-way ANOVA comparing the significance of the differences at different stages inside the same cultivar. A two-way analysis was instead applied to evaluate the difference among cultivars and development stages to the results of the antioxidant tests. Mean separations were performed by application of the Multiple Range Test of Duncan ($p < 0.01$). The correlation coefficients (r) between parameters were calculated using Pearson's coefficient at $p \leq 0.01$ and $p \leq 0.05$. For all analysis, the MSTAT-C software was used.

3. Results

3.1. Dry Matter of Samples

The percentage of berry and leaf dry matter of the cultivars analysed at each sampling time (from 0 to 225 DAF) are reported in Tables 3 and 4.

Table 3. Dry weight expressed in percentage of myrtle leaf extracts.

	Dry Weight of Leaf Samples (%)				
DAF	**Giovanna**	**Grazia**	**Maria Rita**	**Maria Antonietta**	**Sofia**
0	40.76	28.80	42.94	44.88	35.09
30	37.00	40.20	43.11	43.30	44.19
60	41.25	41.00	43.17	43.35	48.32
90	45.20	48.06	46.55	46.11	43.94
120	44.02	46.90	46.43	32.94	50.61
150	39.55	42.84	40.83	42.44	45.46
210	40.59	43.46	44.21	42.77	44.77
225	52.19	38.39	37.30	43.73	42.94

Table 4. Dry weight expressed in percentage of myrtle berry extracts.

	Dry Weight of Berry Samples (%)				
DAF	**Giovanna**	**Grazia**	**Maria Rita**	**Maria Antonietta**	**Sofia**
0	29.22	39.58	29.55	30.85	40.20
30	34.20	28.85	32.03	36.46	39.45
60	37.21	36.71	36.44	38.77	40.41
90	35.52	34.88	35.33	39.36	32.99
120	38.18	34.42	32.94	41.21	34.45
150	21.17	20.53	23.60	27.62	23.65
210	27.04	22.67	28.69	25.61	28.32
225	36.26	31.92	36.39	31.33	34.06

3.2. Total Phenols, Anthocyanins, and Tannins Content

Berry total phenols content was higher during fruit set (0 DAF) than in ripe berry for all cultivars (Figure 2a–e). The following values were observed at 0 DAF: 173.92 mg GAE g^{-1} DW for 'Giovanna', 171.33 mg GAE g^{-1} DW for 'Grazia', 141.23 mg GAE g^{-1} DW for 'Maria Rita', 139.77 mg GAE g^{-1} DW for 'Maria Antonietta', and 145.53 mg GAE g^{-1} DW for 'Sofia'. During berries development, the content of total phenols decreased, with differences among cultivars. The 'Sofia' cultivar showed the lowest content during 225 DAF with 35.45 mg GAE g^{-1} DW (Figure 2f). In the other cultivars, the minimum content was 63.91 mg GAE g^{-1} DW for 'Giovanna' at 210 DAF, 33.46 mg GAE g^{-1} DW for 'Grazia' at 225 DAF, 57.32 mg GAE g^{-1} DW for 'Maria Rita' at 150 DAF, and 52.89 GAE g^{-1} DW for 'Maria Antonietta' at 30 DAF.

In leaf extracts, total phenols content showed during development different trends in the cultivars and a higher content as compared to fruit. 'Maria Antonietta' (Figure 2d) and 'Sofia' (Figure 2e) showed the maximum content at 120 DAF (respectively, with 144.22 and 111.25 mg GAE g^{-1} DW), while the leaves of the Maria Rita cultivar (Figure 2c) reached the maximum content at 225 DAF.

Tannin's content was higher in berries than in leaves. In berries (Figure 2f–l), tannins tended to increase during the first development stages and then decrease in the last stage of ripening in all cultivars, with values between 0 and 22.45 mg CE g^{-1} DW. In 'Giovanna' (Figure 2f) cultivar, the tannins content increases from 60 DAF reaching the maximum content (15.24 mg CE g^{-1} DW) and then decreases to the values of 1.68 mg CE g^{-1} DW in the last stage of ripening. In addition, in 'Grazia' (Figure 2g) and Maria Antonietta (Figure 2i), the maximum values were found at 90 DAF and the minimum content during the overripe stage (225 DAF). Indeed, the 'Sofia' and 'Maria Rita' cultivars reached the highest content of total tannins (Figure 2h, l).

Leaf extracts contained low levels of total tannins during all stages of development with respect to tannins of the berries (Figure 2g–m), with values including 0.28 mg for 'Sofia' and 4.63 mg CE g^{-1} DW for 'Maria Antonietta'.

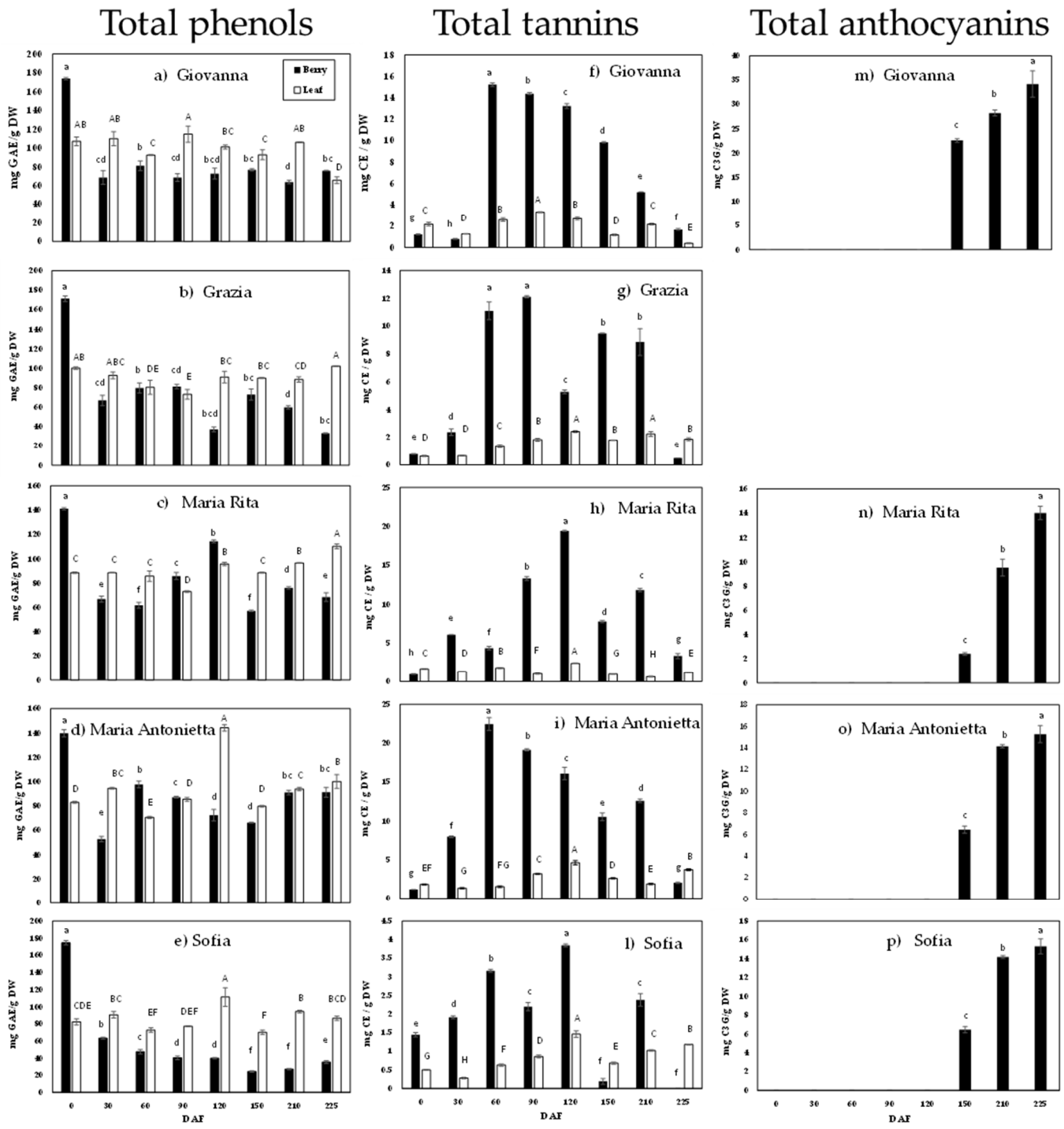

Figure 2. Content of total phenols (**a**–**e**), tannins (**f**–**i**,**l**), and anthocyanins (**m**–**p**) in leaf and berry extracts of the different cultivars. The 'Grazia' cultivar give white fruits. Data are the mean and standard deviation of three replicates. Data labelled with the same letters are not significantly different for components of the berry tissues (lowercase) and leaf tissues (capital) letters according to Duncan's Multiple Range Test at $p \leq 0.01$ level.

Anthocyanins were only detected in the berries of the pigmented fruit cultivars. Anthocyanins increased from 180 DAF to 225 DAF (Figure 2m–p). 'Giovanna' (Figure 2m) was the cultivar with the highest content (34.20 mg C3G g^{-1} DW at 225 DAF). In the other cultivars, the anthocyanins content at 225 DAF were 14.02, 21.64, and 15.28 mg C3G g^{-1} DW, respectively for 'Maria Antonietta', 'Maria Rita', and 'Sofia' (Figure 2n–p).

3.3. Antioxidants Activity (AA) with Colorimetric Assays

The results of AA of leaf extracts detected by colorimetric assays are reported in Table 5. The highest AA with DPPH assay was found in 'Maria Antonietta' cultivar at 0 DAF (Table 5) while the lowest activity was for 'Grazia' unpigmented cultivar in the same stage. Regarding the trend of AA during the two development stages, all cultivars except 'Maria Antonietta' showed the highest AA in the leaves sampled at 120 DAF.

Table 5. Antioxidant activity (AA) in myrtle leaves cultivar evaluated by DPPH, FRAP, ABTS, β-Carotene and EPR assays. Data are the mean and standard deviation of three replicate. Values in the same column labelled with the same letters are not significantly different according to Duncan's multiple range test at $p \leq 0.01$ level.

Cultivar	DAF	DPPH (μmol TE/g DW)		FRAP (mmol TE/g DW)		ABTS (mmol TE/g DW)		β-Carotene (CAA)		•OH Scavenging Activity(mmol GAE/g DW)	
Giovanna	0	25.36 ± 0.21	B	53.63 ± 3.09	c	171.55 ± 0.57	d	0.68 ± 0.22	b	1.52 ± 0.10	abc
	120	21.34 ± 0.33	De	83.24 ± 5.46	a	173.91 ± 3.74	d	1.02 ± 0.42	a	1.99 ± 0.04	a
Grazia	0	25.16 ± 0.70	B	61.97 ± 3.38	bc	174.2 ± 12.83	d	0.60 ± 0.01	b	1.25 ± 0.35	bc
	120	18.88 ± 0.04	F	88.87 ± 5.46	a	188.17 ± 5.83	d	1.15 ± 0.15	a	1.72 ± 0.20	abc
Maria Antonietta	0	21.93 ± 0.14	Cd	71.09 ± 2.41	b	211.50 ± 0.03	c	0.63 ± 0.00	b	1.35 ± 0.50	bc
	120	28.29 ± 0.64	A	84.53 ± 1.97	a	242.6 ± 10.93	b	1.02 ± 0.01	a	1.50 ± 0.06	abc
Maria Rita	0	23.80 ± 0.86	Bc	64.38 ± 6.87	b	182.73 ± 2.70	d	0.59 ± 0.01	b	1.35 ± 0.03	bc
	120	19.94 ± 0.63	Ef	84.58 ± 3.53	a	135.7 ± 10.21	e	1.01 ± 0.00	a	1.71 ± 0.04	abc
Sofia	0	22.94 ± 0.62	Cd	93.25 ± 4.45	a	294.25 ± 8.16	a	0.68 ± 0.06	b	1.21 ± 0.04	c
	120	19.06 ± 0.66	F	84.98 ± 8.08	a	245.03 ± 1.21	b	1.05 ± 0.00	a	1.81 ± 0.06	ab

Determining AA with ABTS assay (Table 5), significant variations between the two stages analysed were found for 'Maria Antonietta', 'Maria Rita', and 'Sofia', detecting the maximum AA at 120 DAF with respect to 0 DAF.

The results with β-Carotene bleaching assay (Table 5) showed that the leaves of all cultivars have a higher AA at 120 DAF. The same trend was found in the cultivars with AA detected with FRAP assay (Table 5), except for 'Sofia' which shows no significant differences between the two stages.

In Table 6, the AA of berry extracts detected with colorimetric assays are reported. With DPPH assay (Table 6), all cultivars showed the higher AA in the colour-breaking and ripe fruit (210 and 225 DAF). The white 'Grazia' cultivar showed the highest AA with respect the other cultivars, with values of 30.07 e 32.74 μmol TE g^{-1} DW. 'Sofia' cultivar maintains during all the development period the lowest AA without significant differences.

The results with FRAP showed a different trend in the cultivars among the analysed stages (Table 6). 'Grazia' and 'Maria Rita' cultivar reached the maximum AA at 225 DAF. In 'Maria Antonietta', the maximum was reached in concomitance with anthocyanins accumulation (150 DAF), while 'Sofia' cultivar reached the highest AA one month after and then decrease with ripening.

The AA detected with ABTS ranged between 45.27 and 196.52 mmol TE g^{-1} DW (Table 6) and was higher at 150 and 210 DAF, except for 'Sofia' cultivar that showed low AA in all stages.

The AA detected with β-Carotene bleaching (Table 6) followed the same trend in all cultivars: values decreased from 60 DAF to ripe stage. In this case, the highest AA was found for 'Maria Antonietta' that showed an antioxidant coefficient (CAA) of 1.08 at 60 DAF.

Table 6. Antioxidant activity (AA) in berries of myrtle cultivars evaluated by DPPH, FRAP, ABTS, β-Carotene, and EPR assays. Data are the mean and standard deviation of three replicates. Values in the same column labelled with the same letters are not significantly different according to Duncan's Multiple Range Test at $p \leq 0.01$ level.

Cultivar	DAF	DPPH (μmol TE/g DW)		FRAP (mmol TE/g DW)		ABTS (mmol TE/g DW)		β-Carotene (CAA)		•OH Scavenging Activity (mmol GAE/g DW)	
Giovanna	60	20.44 ± 0.65	fg	110.77 ± 8.46	d–h	65.54 ± 3.69	i	1.01 ± 0.07	b	0.12 ± 0.04	d
	150	29.66 ± 1.00	b	119.28 ± 9.05	b–e	148.32 ± 8.19	c	0.38 ± 0.02	j	3.89 ± 0.72	c
	210	24.97 ± 0.06	de	114.29 ± 1.11	c–f	121.85 ± 10.5	ef	0.77 ± 0.02	de	4.67 ± 1.00	bc
	225	22.96 ± 0.73	ef	118.97 ± 5.92	b–e	132.40 ± 1.53	de	0.50 ± 0.01	i	0.13 ± 0.02	d
Grazia	60	19.45 ± 0.73	g	100.80 ± 0.01	hi	109.58 ± 8.79	fg	0.90 ± 0.03	c	0.16 ± 0.01	d
	150	30.07 ± 1.80	ab	109.17 ± 2.53	e–h	118.91 ± 11.7	f	0.43 ± 0.01	ij	4.63 ± 0.77	bc
	210	32.74 ± 0.33	a	119.79 ± 1.58	bcd	176.61 ± 7.27	b	0.73 ± 0.02	def	6.64 ± 1.77	a
	225	22.07 ± 1.65	fg	127.12 ± 1.70	ab	71.68 ± 0.87	i	0.74 ± 0.00	def	0.26 ± 0.11	d
Maria Antonietta	60	21.83 ± 0.23	fg	92.29 ± 0.34	ij	62.42 ± 4.30	ij	1.08 ± 0.02	a	0.15 ± 0.04	d
	150	26.16 ± 1.75	cd	123.95 ± 5.08	abc	111.39 ± 0.15	fg	0.71 ± 0.03	efg	3.75 ± 0.28	c
	210	31.12 ± 0.81	ab	101.74 ± 3.48	gh	173.94 ± 9.58	b	0.66 ± 0.02	fgh	5.32 ± 0.82	b
	225	28.90 ± 1.16	bc	88.62 ± 7.38	j	137.46 ± 2.74	cd	0.67 ± 0.04	fg	0.67 ± 0.15	d
Maria Rita	60	23.15 ± 0.65	ef	110.35 ± 0.82	d–h	107.80 ± 2.30	fg	0.80 ± 0.05	d	0.05 ± 0.01	d
	150	25.36 ± 2.36	de	106.64 ± 1.27	f–h	101.71 ± 11.4	g	0.69 ± 0.02	efg	4.76 ± 0.28	bc
	210	26.37 ± 0.36	cd	105.51 ± 1.14	f–h	196.52 ± 3.01	a	0.68 ± 0.01	fg	5.16 ± 0.24	b
	225	20.42 ± 0.01	fg	130.26 ± 9.17	a	108.60 ± 1.40	fg	0.59 ± 0.02	h	0.31 ± 0.06	d
Sofia	60	13.76 ± 1.34	h	110.53 ± 1.00	d–h	88.57 ± 2.84	h	0.96 ± 0.01	b	0.08 ± 0.02	d
	150	15.72 ± 0.88	h	112.98 ± 2.15	def	45.27 ± 5.10	k	0.91 ± 0.02	c	4.22 ± 0.27	bc
	210	14.72 ± 0.97	h	128.77 ± 0.28	ab	50.14 ± 0.33	jk	0.73 ± 0.01	def	4.84 ± 0.57	bc
	225	13.37 ± 1.12	h	111.71 ± 1.08	d–g	49.60 ± 0.90	jk	0.64 ± 0.02	gh	0.26 ± 0.05	d

3.4. Spin Trapping Assay of the •OH Radical

DMPO traps the hydroxyl radicals produced in the Fenton reaction and forms a DMPO-OH adduct, which has a four-line signal with the following hyperfine splitting constants $a_N = a_H = 14.9$ G (Figure 3). When the extract is present in the reaction mixture, the intensity of the DMPO-OH adduct' signal decreases due to competition, for the hydroxyl radical, between the trapping agent and the antioxidants present in the extract (Figure 3).

Leaf extracts own a lower hydroxyl radical scavenging activity than berries extracts. At 0 DAF, the hydroxyl radical scavenging activity of leaf extracts ranged between 121 and 152 mmol GAE/g D.W, and no statistical differences were observed among cultivars. During leaf development, it slightly increased in all cultivars, but in 'Sofia', only the increase was statistically significant.

At 60 DAF myrtle berries belonging to the five cultivars showed quite similar hydroxyl radical scavenging activities, ranging from 0.05 ± 0.01 (Maria Rita) to 0.16 ± 0.01 (Grazia) (Table 6). At the beginning of fruit development, no differences were observed between pigmented and white cultivars. The berries hydroxyl radical scavenging activity increased during development reaching its highest values at 210 DAF in all cultivars. At this developmental stage, the non-pigmented cultivar 'Grazia' showed a hydroxyl scavenging activity higher than the other pigmented cultivars. At the last sampling 225 DAF, the scavenging activity drastically dropped in all cultivars and reached values similar to those observed at the beginning of fruit development.

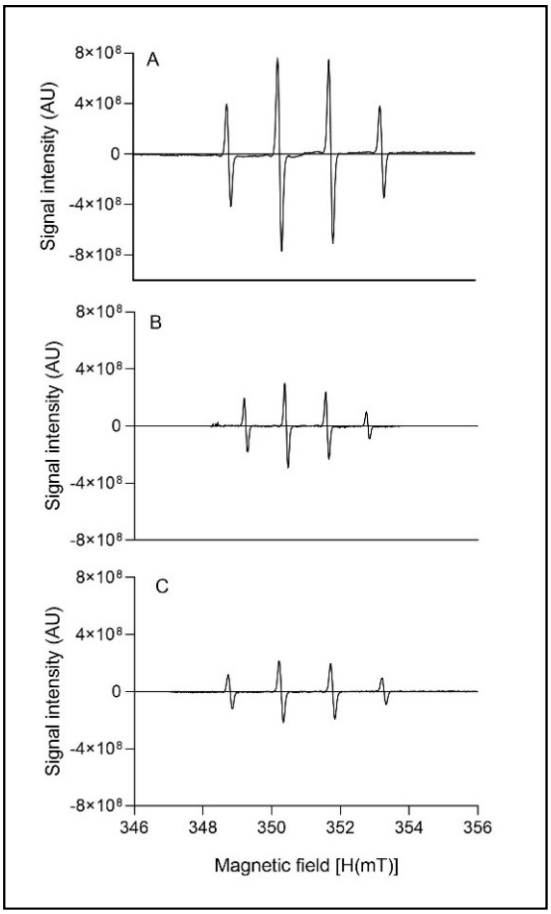

Figure 3. EPR signal intensity of the DMPO-OH radical adduct measured without extract or black line (**A**), with 'Grazia' berry extract (0 DAF) (**B**), and 'Grazia' leaves extract (O DAF) (**C**).

3.5. Correlation between Antioxidant Activity, Phenolic Compounds Content, and Altitude of Site of Origin

The analysis of correlation among the AA detected with the different methods, the content of total phenols, anthocyanins and tannins, and the altitude of the site origin of the cultivar in both tissues (berry and leaves) was carried out.

In leaves extracts (Table 7), the total phenols content was correlated with AA found with DPPH and β-Carotene while the tannins content is involved positively with AA detected with β-Carotene and EPR methods.

Table 7. Correlation coefficient (r) calculated between the phenolic compounds content analysed, antioxidant activity (AA) obtained with the different assays, developmental stages (DAF), and altitude in myrtle berries in leaf extracts. Significance: ** = $p \leq 0.01$; * = $p \leq 0.05$; ^{ns} = not significant.

	Total Phenols	Total Tannins	DPPH	FRAP	ABTS	β-Carotene	EPR
Total tannins	0.719 **						
DPPH	0.507 **	0.288 ^{ns}					
FRAP	0.042 ^{ns}	0.177 ^{ns}	−0.436 **				
ABTS	0.122 ^{ns}	−0.128 ^{ns}	0.157 ^{ns}	0.457 *			
β-Carotene	0.402 **	0.564 **	−0.443 *	0.631 **	−0.047 ^{ns}		
$^{\bullet}$OH scavenging	0.203 ^{ns}	0.368 **	−0.462 *	0.310 ^{ns}	−0.278 ^{ns}	0.673 **	
Altitude	0.041 ^{ns}	−0.319 ^{ns}	−0.139 ^{ns}	0.462 **	0.881 **	0.059 ^{ns}	−0.092 ^{ns}

The total phenols and that of tannins were strictly interrelated in myrtle leaves. With regard to the influences of altitude on AA, the results showed that this parameter influenced positively the antioxidant activity detected with ABTS and FRAP.

Moreover, the AA with DPPH assay is negatively correlated with AA of FRAP, β-Carotene, and EPR assays. The AA detected with ABTS and FRAP assays were correlated positively, as well as the EPR and β-Carotene assays.

Moreover, in berry extracts (Table 8), the total phenols are correlated on the AA (with ABTS and DPPH assays). As found for leaves (Table 7), total phenols were correlated with tannins content in the berry.

Table 8. Correlation coefficient (r) calculated between the phenolic compounds content analysed, antioxidant activity (AA) obtained with the different assays, developmental stages (DAF), and altitude in myrtle berries in berry extracts. Significance: ** = $p \leq 0.01$; * = $p \leq 0.05$; ^{ns} = not significant.

	Total Phenols	Total Tannins	Total Anthocyanins	DPPH	FRAP	ABTS	β-Carotene	EPR
Total tannins	0.698 **							
Total anthocyanins	0.131 ^{ns}	−0.347 **						
DPPH	0.588 **	0.375 **	0.105 ^{ns}					
FRAP	−0.542 ^{ns}	−0.411 **	0.111 ^{ns}	−0.180 ^{ns}				
ABTS	0.546 **	0.228 ^{ns}	0.258 *	0.784 **	−0.147 ^{ns}			
β-Carotene	−0.047 ^{ns}	0.307 **	−0.527 **	−0.452 **	−0.315 *	−0.464 **		
$^{\bullet}$OH scavenging	−0.100 ^{ns}	0.126 ^{ns}	0.058 ^{ns}	0.493 **	0.127 ^{ns}	0.452 **	−0.317 *	
Altitude	−0.571 **	−0.360 *	0.021 ^{ns}	−0.647 **	0.037 ^{ns}	−0.566 **	0.264 *	−0.035 ^{ns}

The results showed that the tannins contribute more to AA than anthocyanins content, although the coefficient of r was not high. Total tannins were correlated with AA detected with DPPH and β-Carotene assays.

The anthocyanins content was weakly correlated to ABTS and β-Carotene. The altitude affected the AA of berries in different manner with respect to myrtle leaves; in fact, the AA (detected with DPPH and ABTS assays) were negatively correlated with the altitude of site of origin, except for the AA with β-Carotene that was weakly positively correlated. Moreover, also the content of total phenols and tannins in myrtle berry decrease with high altitude.

4. Discussion

4.1. Differences in Phenolic Compounds Determined by Biological Diversity of Samples

The recent health claims encourage the consumption of nutraceutical and functional food like myrtle due to the role that phenolic compounds have on the reduction of degenerative diseases [42–46].

Myrtle is a natural source of bioactive compounds [47] exploitable for medical and pharmaceutical purposes. The domestication process of Sardinian myrtle genotypes provided cultivars with high biomass production; these were partially studied for leaf and fruit phenolic profile; however, little is known about the evolution of these compounds during fruit and leaf development [1,16,22,48].

The results of this paper widen the information on myrtle plant antioxidant properties and suggest some criteria for the selection of biomasses with high medical and pharmaceutical potentialities.

The concentration of the main phenolic sub-groups in myrtle leaves and berries was evaluated during fruit and shoot development. As previously observed, the total phenolic content of myrtle berries decreased during fruit maturation [33,38]. This was observed both in berries of 'Giovanna' (pigmented) and 'Grazia' (unpigmented), two cultivars with the same site of origin.

As found by Snoussi et al. [49], the total phenols of ethanol myrtle extracts were higher in berry than leaf. The changes of phenolic concentration during leaf development were different among cultivars, but the final value was similar to other small berries [50].

Pigmented berries synthetized anthocyanins from colour-break stage (150 DAF) until full ripe according to other authors [38]. In all small fruit, the high anthocyanin concentration is associated to favourable effects on human health [51].

Myrtle pigmented berries are a good anthocyanin source, and particularly 'Giovanna' showed the highest concentration. Some authors indicated an anthocyanin content of 8.4–41.1 mg C3G/g DW in haskap berries [52], 15.4 mg of cyanidin in the cultivar 'Red thorn' of blackberry at ripe stage [53], and 14.5 mg of cyanidin in rabbiteye blueberry fresh berries [54].

The unripe myrtle berry has an astringent taste due to the high tannin concentration, which decreased when anthocyanin storage began.

In our previous study, we observed a genotype dependent decrease of tannin concentration; moreover, the highest tannin concentration was detected at 30 DAF and suddenly disappeared at the subsequent stages, contrary to what was observed in this work [38].

In this paper, myrtle leaves had less tannins than berries, contrary to what was observed by other authors [55]. Both berries and leaf tannins concentrations were positively correlated to total phenols (respectively, r = 0.698 and r = 0.719).

The correlation analysis revealed no effect of altitude on leaf total phenols, whereas a negative correlation was calculated with berries total phenols and tannins concentrations.

These results suggest the involvement of other factors associated to altitude, thermic conditions or increased exposition to solar radiation, in the accumulation of secondary metabolites. These factors may induce plant stress with a consequent decrease of tannins and total phenols.

4.2. AA Observed According to Biological and Sampling Variables

The leaf AA analysed at two development stages showed a high variability among genotypes, tissue development, and method used to evaluate AA.

The results of the DPPH, Frap, and β-carotene-bleaching methods showed an increase of the leaf extracts AA in all cultivars ('Maria Antonietta' is the only exception) during leaf development. Instead, with ABTS method, the AA of leaf extracts reached the maximum AA in autumn season (0 DAF).

A previous study [32] on the AA of Algerian myrtle (*M. communis* var. *italica* L.) leaves and berries at two stages of development (September and December) revealed a higher free radical scavenging in tissues sampled in September.

In addition to the colorimetric assays, the spin trapping method coupled with EPR spectroscopy was used to estimate the antioxidant activity of both leaves and berries extracts. In a previous paper, this methodology was employed to study the hydroxyl radical scavenging activity of myrtle berries hydroalcoholic extracts used for the production of the typical liqueur [34]. The results indicated that the higher the ethanol concentration in the extraction medium, the higher the hydroxyl radical scavenging activity of the hydroalcoholic extracts.

The concentration of total phenols and tannins affected the AA of myrtle leaves. Total phenols were correlated to AA detected with ABTS and β-Carotene methods while tannins content influenced β-Carotene and hydroxyl radical scavenging activity results. According to Sanna et al. [34] the high hydroxyl radical scavenging activity of myrtle hydroalchoolic extracts was likely due to the high concentration of ellagic acid in the extracts even after 10 months of storage. The results presented in this paper seem to confirm this hypothesis since the hydroxyl radical scavenging activity was positively correlated to tannins concentration in myrtle leaf extracts. Moreover, no differences were observed between the sampling dates in leaf extracts, with the only exception of the cultivar 'Sofia'.

Amensour et al. [18] and Pereira et al. [56] reported a higher antioxidant activity in leaf extracts than berries ones, suggesting that leaf phenolic composition has a higher antioxidant power than anthocyanins, principally present in berries.

The AA of the berries was assessed during four development stages, from unripe (60 DAF) to overripe fruit stage (225 DAF). The 150 DAF correspond to fruit colour-break. AA results showed a high variability according to genotype and fruit development. The ABTS and DPPH radical scavenging activities were highest, in all cultivars at 150 DAF and 210 DAF (ripe stage fruit). Only, the AA of 'Sofia', the cultivar originated from the locality with highest altitude, showed any variation during the development stages of fruit.

Only with the β-carotene assay, the AA was higher in the fruit when is unripe with respect the ripe stages. The ability of scavenging the OH radicals, evaluated by EPR, indicated higher values at 150 and 210 DAF for all berry cultivars.

In black chokeberry (*Aronia melanocarpa*) the antiradical activity was greater in unripe fruit compared to ripe one [57]. In sweet cherry, the evolution of antioxidant activity during 14 developmental stages showed an increase alongside anthocyanins accumulation. In strawberry fruit, the antioxidant activity at extra-early period during was lower of 17.8% than early and ripe stages [58].

In this study, the unpigmented genotype had higher DPPH and ABTS radical scavenging activities than pigmented ones. Messaoud and Boussaid [59], comparing white and pigmented myrtle Tunisian genotypes, found a greater AA in pigmented berries than in white ones. These authors calculated an IC_{50} value of 2.1 and 2.8 mg/mL for pigmented and white genotype with DPPH assay, and the value of 2.7 mmol Fe^{2+}/g for pigmented berries and 2.1 mmol Fe/g for white berries.

The content of phenolic compounds, anthocyanins, and tannins affected the AA of myrtle berries in a different way. The total phenol content influenced the AA with ABTS (r = 0.546) and DPPH (r = 0.588) assays, while the tannins are positively involved in AA with β -carotene (r = 0.307), and negatively with AA with FRAP assay (r = −0.411). Dudonne et al. [60], analysing aqueous extract of 30 medicinal plants, observed a correlation between the antioxidant activity obtained with DPPH, ABTS, and FRAP assays and total phenol content.

The anthocyanins content in this study appears to play a more marginal role in the AA of myrtle fruit than the total phenols and tannins. It is correlated with ABTS (r = 0.258) and negatively with β -carotene (r = −0.527). Contrary to our results, anthocyanins of grape extract were highly correlated with FRAP, DPPH, and ABTS methods [61]. In other fruit, high amounts of anthocyanins were correlated with AA obtained with DPPH assay [62]. Arnous et al. [62] observed a greater impact on antioxidant activity of total phenols and total flavanols than anthocyanins in Greek red wines.

4.3. AA as Influenced by the Altitude of the Site of Origin of Cultivars

Another objective of this study was to evaluate the influence of altitude, and consequently related environmental parameters, of the site of origin of cultivars on AA despite the actual cultivation in the same field of comparison. We found a different influence of the altitude on AA according to the tissue of plant. The altitude was positively correlated with AA (detected with FRAP and ABTS) of leaf extracts; however, different correlations between AA (with ABTS and DPPH) were found for berry extracts. In this case, the assay used for AA evaluating strongly affected the results, thus confirming the previously observed variability. Various climatic factors depend on altitude like visible radiation, UV radiation and air temperature [63]. Altitude affects the natural selection of plants genotype, of bioactive compounds, plants' physic-chemical characteristics, and sensory properties [64]. There is no agreement in the literature on the effects of altitude on antioxidant activity. Most of the papers deal with the influence on phenotypic expression rather than on antioxidant activity. Gündüz and Özbay [65] observed in some strawberry genotypes an increase of antioxidant activity with increasing altitude, although the greater variability in this activity was due to the genotype. Another study on this species indicated

a lower AA in the cultivar grown at 1500 m above sea level site than 1100 and 1200 m sites, due to the lower accumulation in anthocyanins at high altitude [66]. The ability of radical scavenging in *Calluna vulgaris* was higher with rising altitude due to the higher flavonoid's accumulation.

As a completion of our study, the promotion of a further experiment with the same genotypes growing at different altitudes appears logical, including the localities of origin of the cultivars. This additional experiment will be expected to show how the genetic differences may express some eventual plus variance that the actual location of the field of comparison is unable to stress.

4.4. Information Provided by the Application of Different AA Methods

The methods we have used allow us to assess the overall antioxidant activity of plant tissues, considering some of the main phenolic chemical families, without evaluating the contribution of each individual compound (e.g., delphinine or gallic acid) to this activity. This is undoubtedly a possible perspective to improve the information obtained by our experiment, because the AA is dependent on each individual compound role within the main group (e.g., flavonoids, tannin families) and by their chemical modifications [67]. For example, the glycosylation of cyanidin anthocyanin increases its antioxidant activity while in malvidin anthocyanins, the antioxidant activity decreases after glycosylation [68]. Arnous et al. [62] suggest that a low correlation between total content of anthocyanins and antioxidant activity may be justified by both monomeric and polymeric anthocyanins, characterized by different antioxidant activities.

However, the results of the antioxidant activity presented in this paper provide complete information about the ability of myrtle extracts to counteract the damaging effects of free radicals. Several authors reported the importance of the use of different methodologies to determine the antioxidant properties of plant extracts since each method reflects different mode of action of the antioxidant molecules and may provide different information about the extract's activity and use.

Myrtle extracts used as food additives protect food from lipid oxidation while as food ingredient; they protect cells and tissues from oxidative damage. In this paper, we compared the results of the antioxidant activities measured with different methods with the aim to provide a wide spectrum of information on myrtle extracts' antioxidant activity. The methods used in this paper were based on the antioxidants' reaction mechanisms: DPPH (free radical scavenging activity), ABTS (radical cations reducing activity), FRAP (metal ions reducing activity), the β- carotene bleaching method (peroxyl radical scavenging activity), and spin trapping method coupled with EPR (hydroxyl radical scavenging activity).

DPPH and ABTS are the most widely used methods to determine the antioxidant activity of plant extracts and often used for comparison purposes. In myrtle, leaf extracts DPPH and ABTS were used to study the radical scavenging properties of Sicilian myrtle populations to provide a chemical characterization of the leaves for future domestication programmes [69]. It is not always easy to compare results from different laboratories since they are often reported with different units of measurement. The cultivars analysed in this paper presented a high variability of the radical scavenging activities measured with DPPH and ABTS methods, and on the contrary, a moderate variability was observed among Sicilian populations.

Regarding ABTS method, the ability of the antioxidants to reduce the radical cation ABTS+ depends on their reduction potential. Only the antioxidants with a redox potential lower than that of the couple ABTS●+/ABTS (E0 = 0.68 V) are able to reduce the ABTS●+ radical [70]. Similarly, the FRAP method measures the reducing power of the extracts. It is based on the ability of the extracts to reduce the yellow ferric tripyridyltriazine complex (Fe (III)-TPTZ) to the blue ferrous complex (Fe (II)- TPTZ). This method provides similar results to ABTS assay since the redox potential of the couple Fe (III)/Fe (II) is 0.7 V similar to that of ABTS●+/ABTS couple. The lack of significant correlation observed in this paper between results obtained from ABTS and FRAP methods may be due to the effect of a

different reaction environment on the extracts: in particular, the pH value. The FRAP assay was carried out under acidic conditions (pH 3.6), while ABTS under neutral conditions (pH 7.4). Moreover, in the extracts, other electron donating substances with an E0 lower than that of the couple Fe (III)/Fe (II) may cause an overestimation of FRAP results.

The β- carotene bleaching method estimates the ability of plant extracts to scavenge peroxyl radicals, which are the major causes of food quality deterioration. It is based on the ability of the antioxidants present in the extracts to react with the peroxyl radical thus preventing the β-carotene bleaching. In the case of myrtle, this test may provide a good indication of the evolution of leaf tissue that in the same season showed a natural increase of the chlorophyll and related carotenoid content, which can contribute to the observed increase of the AA from springtime to autumn.

The spin trapping method coupled with EPR is the most specific and reliable method for the estimation of biological relevant radicals like hydroxyl and superoxide radicals. The hydroxyl radical is one of the most dangerous radicals produced in cells; the spin trapping method provides useful information about the biological relevance of extracts. In food chemistry, the spin trapping method has been used to evaluate the antioxidant activity of food, beverages, and plant extracts [21,22,34].

5. Conclusions

Numerous studies highlight the potential use of the myrtle plant for medicinal purposes. Clinical studies carried out so far show a key role for the AA of phenolic compounds of myrtle on these health-promoting properties.

In this study, the influence of the genotype, biodiversity of the samples, sampling times, altitude of site of origin on the AA in myrtle berry and leaf extracts were evaluated. We used both spectrophotometric and spectroscopic methods to evaluate the AA of the extracts with different mechanism of action.

With respect to development stages of samples, the trend of AA in leaf and berry extract was different among the cultivars and the method used for the AA evaluation. The evaluation of development stage of vegetal material is necessary to identify the best harvest time to maximize the AA.

In leaf extracts, the total phenols content was correlated to the AA detected with ABTS and β-Carotene assays while the content of tannins content was correlated to β-Carotene and EPR. In the berry extracts, the content of phenolic compounds, anthocyanins, and tannins affected differently the AA. The total phenols content influenced the AA with ABTS and DPPH, while the tannins are positively involved in AA with β-Carotene and negatively to AA detected with FRAP assay. Instead, the anthocyanins of the berries were correlated positively with the AA with ABTS and negatively with the AA with β -carotene, showing a lower contribution to the AA of myrtle berries. The role of other flavonoids compounds such as tannins should be studied in depth.

The altitude of the site of origin of cultivars affected positively the AA in leaves and negatively the AA in berries but with a high variability in the results due to the type of assay used. Subsequent studies on a larger number of cultivars and other climatic parameters, as temperatures and rainfalls, would be useful to better understand both the mechanism of phenolic compound accumulation and antioxidant activity. In addition, it would be interesting to evaluate the behaviour of these cultivars with respect to these traits in their areas of origin to evaluate the differences with the same growing conditions.

In conclusion, these genotypes have shown good antioxidant activity and will subsequently be assessed for their biological properties. In particular, these genotypes will be tested for their possible use in the formulation of food supplements or pharmaceutical products, using processing plant residues as a source of bioactive compounds.

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