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Evolution of Phytochemical Variation in Myrtle (*Myrtus communis* L.) Organs during Different Phenological Stages

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Abstract: Myrtle (*Myrtus communis* L.) is an important medicinal plant, of which all parts have pharmaceutical and nutritional applications and which is distributed throughout the southwest regions of Iran. The aerial parts of myrtle were collected at different phenological stages (vegetative, flowering, unripe fruit and ripening fruit stages, after the fully mature fruit dispersal stage) from Fars Province of Iran and were analyzed with GC–FID and GC–MS. All the data were submitted to multivariate statistical analysis, showing many differences among the various plant parts and their phenological stages. Monoterpene hydrocarbons (18.9–50.5%) and oxygenated monoterpenes (38.2–72.4%) were the most abundant class in the volatile emissions, with α-pinene, 1,8-cineole, limonene, linalool, α-terpineol, linalyl acetate and geranyl acetate being the main constituents in the majority of the examined samples. α-Pinene content ranged from 8.88% at the after fully mature fruit dispersal stage (leaves) to 32.84% at the flowering stage in the leaves. Limonene, which is the dominant component at the flowering stage, was 23.97% in the flowers, while the limonene amount was 0.14–1.42% at other harvest stages. The greatest 1,8-cineole amount was obtained prior to flowering, 45.98%, while the lowest amount was obtained at the flowering stage, in the flowers. The total phenol and flavonoid contents varied between different myrtle parts: the highest total phenol and flavonoid contents were obtained for the extract made of the before-flowering stage in leaves (66.52 mg GAE/g and 7.49 mg QE/g extracts). Overall, the study indicated that collecting myrtle at the vegetative and flowering stages, especially the leaves organs, would be of considerable importance.

Keywords: wild collection; aromatic profile; phytochemical composition; growth stages

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Citation: Hazrati, S.; Hosseini, S.J.; Ebadi, M.-T.; Nicola, S. Evolution of Phytochemical Variation in Myrtle (*Myrtus communis* L.) Organs during Different Phenological Stages. *Horticulturae* **2022**, *8*, 757. https://doi.org/10.3390/horticulturae8090757

Academic Editor: Alessandra Durazzo

Received: 21 July 2022
Accepted: 19 August 2022
Published: 23 August 2022

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1. Introduction

Myrtaceae is one of the largest plant families, with 145 genera and 5970 species, distributed throughout the tropical regions. The genus *Myrtus* includes flowering plants with three species reported in areas of the Middle East and Asia. Myrtle (*Myrtus communis* L.) is one of the most important aromatic and medicinal species from this family. This aromatic plant is native to Southern Europe, North Africa and West Asia and is widely grown and distributed in the South and Southwest of Iran. It is also cultivated in some gardens [1,2]. It is an evergreen shrub reaching a height of 1.8 to 2.4 m [3]. The leaves are opposite, almost without petioles, ovate-lanceolate, fully acuminate, coriaceous, durable, glabrous and glossy. They are covered with clear spots with unspecified nervures, having white, singular, fragrant-smelling flowers, which are quite large, and peduncles with various anthers. The berries are called "mursins" and are used as a flavoring in the Middle East [4]. The berries are almost fleshy, ovoid-ellipsoid with dark-blue or black-blue coloring and different seeds.

Various organs of this plant are utilized in the pharmaceutical and cosmetic industries [5–7]. Myrtle has a long history in traditional Iranian medicine (TIM), where the leaf and berry are used against infectious diarrhea, cough and constipation and to improve oral wound healing [8]. It is reported to have hypoglycemic, antihemorrhagic and appetizing properties and is also used externally for wound healing [9,10]. Studies on myrtle have shown high antiviral [11], antioxidant [12], antimicrobial [13], antifungal [14] and anticancer [15] properties. Insecticidal [16], disinfectant [17,18], analgesic [4] and anti-hyperglycemic [19] properties were also reported.

The different organs of the plant contain fibers, sugars, fatty acids and many components with antioxidant properties. Essential oils (EOs), phenols, flavonoids and anthocyanins are some of the most significant components of myrtle [20,21]. The EOs are obtained from various organs of the plant and bear different biological properties [10]. Phytochemical compositions of different plants are influenced by their phenological growth stages. The concentration of phytochemical compounds in the same stages of development varies depending on the organs of the plant. The function of the produced EO quality depends on the vegetative region, the harvest season, and the organs utilized [22–25]. Additionally, the content of the EO is different in various dry organs, and it is between 0.4% and 0.5% in leaves, 0.4% in flowers, 0.5% in the unripe fruits, and 0.02% in the ripe fruits [20,26].

Some studies have been carried out on the EO composition of myrtle, showing that the most significant EO compounds consist of terpinolene, 1,8-cineole, linalool, linalyl acetate, α-pinene and limonene [26–29]. The EO content in medicinal and aromatic plants has different biological functions, which depend on different factors such as genotype, environment, plant organ, harvest season and the stage of the phenological development [30–33]. In several studies, biological activity of the EO of myrtle has been shown to change at different vegetative stages in the various organs [31,34]. The plant-derived secondary metabolites, such as phenolic contents, have the potential to scavenge the free radicals, which exist in all different parts of the plant, such as in leaves, fruits and seeds [35–39].

The phenolic components are also important compounds in myrtle and have been studied [40–42]. Many studies have shown that myrtle can be utilized as a source of antioxidants [17,20,40,43,44]. Since myrtle has medicinal, nutritional, industrial and cosmetic properties, the functions and applications of its various organs, such as its leaves and fruit, are increasing in various industries [27].

Several studies have been conducted on the phytochemical compounds of myrtle. These compounds are important and are widely used in a variety of industries. It is important to mention that no research has yet been presented on the variations in phytochemical compounds (EO content and composition, total phenol and flavonoid contents) and their effect on the levels of antioxidant properties of the various organs of myrtle at different phenological stages. Thus, the objective of this study was to examine the phytochemical composition of myrtle at different phenological stages and in different organs, as well as to determine whether there is any correlation between antioxidant activity and total phenolic and total flavonoid contents.

2. Materials and Methods

2.1. Plant Material and Sampling

The current study took place in spring, summer and winter of two years, 2017 and 2018, in the city of Kazerun, Fars Province, Iran, 29°46′38.64″ N and 51°34′12.36″ E, 860 m asl, collecting myrtle organs in different samplings. The first sampling started 30 days after the onset of vegetative growth, in spring, collecting leaves only. The following samplings were carried out at different stages including before flowering, collecting leaves (5 May 2017); at the flowering stage, collecting leaves and flowers (5 June 2017); at the unripe fruit stage, collecting leaves and unripe fruits (20 August 2017); at the ripe fruit stage, collecting leaves and ripe fruits (6 November 2017) and after the fully mature fruit dispersal stage, collecting leaves only (20 March 2018) (Figure 1). All samplings were randomly selected

from 10 myrtle bushes. From every stage and each organ ca. 1 kg of material was collected and divided into three replications. In all samples, the organs were separated from each other after recollection and were dried in shady conditions at a temperature of 25 °C for three weeks.

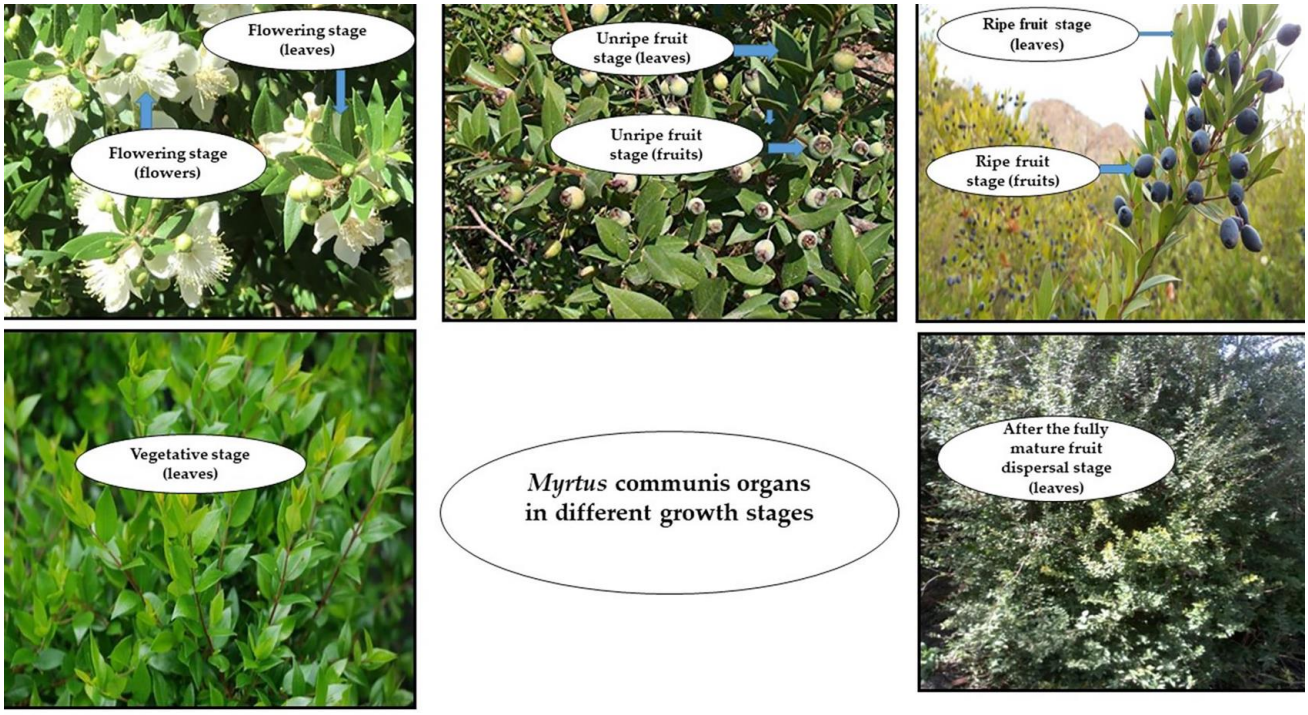

Figure 1. *Myrtus communis* organs in different phenological stages.

2.2. EO Isolation

Every sample of the organs was chopped using a blender at low speed, and 100 g of the dried sample of different organs including leaves, fruits and flowers was subjected to hydro-distillation for 3 h using a Clevenger apparatus. For every organ, three distillations were performed. Following this, the samples were dehydrated using Na_2SO_4, and the EO content was estimated for each organ. The EOs were stored in sealed vials at −20 °C until the chemical analysis.

2.3. EO Analysis

The EOs were analyzed using a gas chromatography–mass spectrometry device (Thermoquest Finnigan) and a 60 m × 0.25 mm, 0.25 µm fused silica column (HP−5). The oven temperature was initially at 60 °C, increased by a ramp-up of 5 °C/min for 38 min and then kept constant at 250 °C for 10 min. Helium was used as a carrier gas with a flow rate of 1.1 mL min^{-1}. The splitting ratio was 1:100, and the injector and detector temperatures were adjusted to 250 and 280 °C, respectively. The ionization voltage, scan time and mass range were 70 eV, 0.4s and 40–300 m/z, respectively. The EO constituents were determined using retention indices as well as Wiley and NIST 11.0 mass-spectral libraries and the previous literature. The percentage of the compounds was calculated by electronic integration of FID peak areas without the use of response factor correlation. The percentage evaluation of the oil components was carried out by performing area normalization.

2.4. Preparation of Extracts

To measure the phenolic and flavonoid contents of the samples (three replicates for every organ), the alcoholic extracts were prepared by soaking 0.2 g of the powdered plant in 25 mL of 80% aqueous methanol (*v*/*v*) for 24 h followed by filtration using Whatman filter paper no. 1 at 25 °C. Then, the filtrates were evaporated under a fume hood (Fater

Electronic, CH612, Tehran, Iran) for 48 h. Afterward, they were weighed and then stored in a freezer at −20 °C.

2.5. Determination of Total Phenolic Content

The total phenolic content of each extract was determined using the Folin–Ciocalteu method [45]. Briefly, 200 µL of crude extract (10 mg/mL) WAS mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent (Merck, Kenilworth, Germany) for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate (Sigma-Aldrich, Darmsdats, Germany). After incubation for 30 min at room temperature, the absorbance was measured with a UV–Vis spectrophotometer (PG Instruments) at 750 nm. Absorbance values were compared against a standard curve of gallic acid (Sigma-Aldrich, Darmsdats, Germany), and the total phenolic content was expressed in terms of milligrams of gallic acid equivalent (mg GAE)/g dry weight of plant.

2.6. Determination of Total Flavonoid Content

Total flavonoid content of each extract was determined according to an aluminum chloride colorimetric assay [46]. Briefly, 50 µL of extract (1 mg/mL) was mixed with 10 µL of 10% aluminum chloride (Sigma-Aldrich, Darmsdats, Germany) solution and 10 µL of 1 M sodium acetate (Sigma-Aldrich, Darmsdats, Germany). After 15 min of incubation, the absorbance was measured at 415 nm. Total flavonoid content was expressed as quercetin equivalent per gram dried weight (mg QE/g DW extract).

2.7. Antioxidant Activity (DPPH Radical Scavenging Assay)

The antioxidant activity of extracts was measured with the DPPH (2, 2′-diphenyl−1-picrylhydrazyl, Sigma-Aldrich, Darmsdats, Germany) free radical scavenging method as described by Akroum et al. [47]. Radical scavenging activity was gauged based on the following equation:

$$\text{Percentage of radical scavenging activity} = (\text{Abs control-Abs sample})/\text{Abs control} \times 100$$

2.8. Statistical Analysis

All data were analyzed in triplicate with an experiment based on a randomized complete block design. The data were analyzed using Statistical Analysis System (SAS) software 9.2 and compared using one-way ANOVA. Data were recorded as mean ± SE. Significant differences between means were determined by Duncan's test, and p values < 0.05 were regarded as significant. Correlation analysis was carried out with the Pearson method based on phytochemical characteristics. Moreover, principal component analysis (PCA) was performed using XLSTAT Version 2018.1 software (Addinsoft, Paris, France).

3. Results and Discussion

3.1. EO Content

The ability of medicinal plants to produce EOs varies with their phenological stage [48]. For pharmaceutical, food and cosmetic applications, breeders must consider the proper harvest time to achieve the best yield. For this reason, the EO content of various organs was studied in different phenological growth stages of myrtle. The EO contents from leaves, flowers and fruits and their variation according to the phenological stage are reported in Figure 2. According to the results, the highest EO content was achieved in leaves at the flowering stage of the plant (1.25%). The same result was obtained in the leaves after the fully mature fruit dispersal stage, while the lowest amount was obtained in fruits at the ripe fruit stage of the plant (0.35%). According to the results, high levels of diversity were observed in EO content in the various organs at different phenological stages of myrtle (Figure 2).

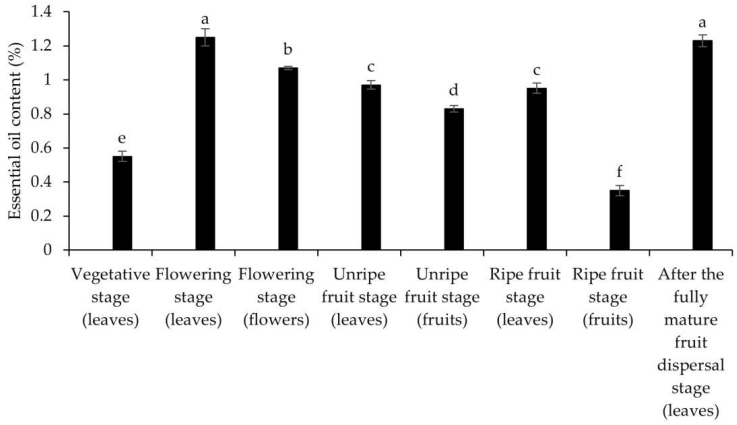

Figure 2. Changes in essential oil content (% *w*/*w*) of myrtle organs in different phenological stages. Essential oil contents with different subscript were significantly different at $p < 0.05$ (Duncan test).

Variations in EO content observed for leaves, flowers and fruits collected from the same zone during different growth periods suggest that in addition to physiological changes occurring within organs, EO biosynthesis is also affected by interactions between these organs and structural and functional modifications at the apical apex of branches. By converting the apical branch meristem to an inflorescential meristem, EOs are secreted more often [49]. Thus, collecting leaves at the flowering stage and after the fully mature fruit dispersal stage may ensure the highest levels of EO. Developmental periods are marked by a reduction in EO due to the accumulation of photosynthetic products in the endosperm [50].

Other researchers have also reported similar differences in the EO content in the various organs of myrtle [20] and at the different vegetative stages of the fruit [26]. EO was found to be most concentrated during flowering, and, as a result of fruit development, the amount was reduced. This supports the results of the current study. In previous studies, the EO amount was 0.4–0.5% in leaves, 0.4% in flowers, 0.5% in unripe fruit and 0.02% in ripe fruit [5,20]. However, there has not been any research published verifying the amount of EOs in different organs at different growth stages. These variations could be the cause of the interaction between the physiological activities of the plant at different vegetative stages and the environment. Biological and genetic variants, environmental changes, soil properties, seasonal changes and light changes cause the amount of EO to be different in different organs and phenological stages. Although the production of EO in aromatic plants is guided by genetic processes, it is significantly influenced by environmental factors such as temperature, light and relative humidity; thus, these factors cause changes in the growth and the quantity and quality of their phytochemical compounds [51–53]. The content of water and soil nutrients and minerals as well as nitrogen play a significant role in the chemical composition and quality of EOs [54–56]. Because during the growth period of the plant the characteristics of the soil around the roots may change, the diversity in terms of production of EO at different phenological stages seems reasonable.

3.2. EO Composition

Fluctuations in EO composition of the myrtle leaves, flowers and fruits at different growth stages are presented in current study: 23 compounds in the various organs were observed at different phenological stages of myrtle, representing 92.97 to 97.78% of the total compositions (Table 1). The results showed that there is a meaningful difference in the EO composition in the various organs at the different phenological stages, indicating that the EO composition also depends on the plant organs and collection time. The dominant compounds of the EO in the various organs at the different phenological stages consisted of α-pinene, 1,8-cineole, limonene, linalool, α-terpineol, linalyl acetate and geranyl acetate. Other researchers have reported the same dominant compounds in different populations and organs of myrtle [1,10,20,26,57–59].

Table 1. EO composition (%) of different myrtle organs in different phenological stages.

No.	Component	RI	Vegetative Stage (Leaves)	Flowering Stage (Leaves)	Flowering Stage (Flowers)	Unripe Fruit Stage (Leaves)	Unripe Fruit Stage (Fruits)	Ripe Fruit Stage (Leaves)	Ripe Fruit Stage (Fruits)	After the Fully Mature Fruit Dispersal Stage (Leaves)
1	Isobutyl isobutyrate	911	0.12 ± 0.00^{a}	-	-	-	-	0.10 ± 0.00^{a}	0.04 ± 0.02^{b}	0.10 ± 0.00^{a}
2	α-Thujene	927	0.12 ± 0.01^{a}	0.12 ± 0.00^{a}	0.14 ± 0.00^{a}	0.12 ± 0.00^{a}	0.15 ± 0.01^{a}	0.11 ± 0.01^{a}	0.11 ± 0.01^{a}	0.05 ± 0.03^{b}
3	α-Pinene	938	25.03 ± 0.62^{b}	32.84 ± 0.32^{a}	19.01 ± 0.58^{c}	19.42 ± 0.72^{c}	15.00 ± 2.08^{d}	10.09 ± 0.61^{e}	11.50 ± 2.15^{e}	8.88 ± 0.33^{e}
4	β-Pinene	979	1.32 ± 0.36^{a}	0.60 ± 0.01^{b}	0.34 ± 0.01^{b}	0.52 ± 0.08^{b}	0.10 ± 0.00^{b}	0.27 ± 0.02^{b}	0.43 ± 0.22^{b}	0.65 ± 0.26^{b}
5	δ−3-Carene	1013	0.78 ± 0.33^{a}	0.65 ± 0.05^{a}	0.18 ± 0.01^{b}	0.18 ± 0.01^{b}	0.20 ± 0.01^{b}	0.13 ± 0.01^{b}	0.11 ± 0.01^{b}	0.25 ± 0.03^{b}
6	p-Cymene	1028	0.98 ± 0.51^{a}	1.00 ± 0.05^{a}	0.20 ± 0.01^{b}	0.43 ± 0.05^{ab}	0.20 ± 0.03^{b}	0.32 ± 0.01^{b}	0.24 ± 0.01^{b}	0.17 ± 0.01^{b}
7	Limonene	1031	1.42 ± 0.12^{b}	0.80 ± 0.06^{c}	23.97 ± 0.09^{a}	0.85 ± 0.18^{c}	0.64 ± 0.31^{cd}	1.07 ± 0.08^{bc}	0.14 ± 0.01^{e}	0.33 ± 0.11^{de}
8	1,8-Cineole	1035	45.98 ± 2.50^{a}	33.22 ± 0.98^{c}	12.56 ± 0.26^{f}	22.77 ± 0.38^{d}	16.01 ± 0.15^{e}	38.52 ± 0.37^{b}	34.74 ± 0.20^{c}	44.54 ± 0.85^{a}
9	γ-Terpinene	1060	0.37 ± 0.14^{b}	0.11 ± 0.00^{c}	0.11 ± 0.01^{c}	0.19 ± 0.01^{bc}	1.34 ± 0.13^{a}	0.15 ± 0.01^{c}	0.13 ± 0.01^{c}	0.20 ± 0.01^{bc}
10	Linalool	1106	8.12 ± 0.01^{e}	10.56 ± 0.27^{d}	16.14 ± 0.64^{c}	15.54 ± 0.35^{c}	16.18 ± 1.08^{c}	23.37 ± 0.01^{a}	17.39 ± 0.92^{c}	19.59 ± 0.47^{b}
11	α-Terpineol	1198	3.57 ± 0.25^{e}	5.74 ± 0.16^{d}	8.45 ± 0.03^{b}	15.17 ± 0.81^{a}	8.34 ± 0.16^{b}	8.90 ± 0.49^{b}	6.15 ± 1.38^{cd}	7.73 ± 0.22^{bc}
12	Methyl chavicol	1206	0.62 ± 0.29^{bc}	1.54 ± 0.05^{a}	0.90 ± 0.06^{b}	0.78 ± 0.19^{b}	0.66 ± 0.03^{bc}	$0.48 \pm 0.08^{b\text{-}d}$	0.27 ± 0.03^{cd}	0.16 ± 0.01^{d}
13	β-Citronellol	1237	0.41 ± 0.12^{b}	1.22 ± 0.38^{a}	0.16 ± 0.02^{b}	0.67 ± 0.12^{b}	0.41 ± 0.08^{b}	0.56 ± 0.08^{b}	0.23 ± 0.02^{b}	0.36 ± 0.12^{b}
14	Linalyl acetate	1257	2.79 ± 0.28^{c}	2.71 ± 0.35^{c}	2.45 ± 0.03^{c}	6.86 ± 0.51^{a}	5.57 ± 0.75^{b}	5.18 ± 0.08^{b}	3.22 ± 0.31^{c}	4.96 ± 0.00^{b}
15	Methyl citronellate	1262	0.77 ± 0.44^{cd}	2.04 ± 0.02^{b}	0.18 ± 0.01^{d}	2.62 ± 0.28^{b}	5.04 ± 0.34^{a}	0.31 ± 0.03^{d}	5.00 ± 0.08^{a}	1.19 ± 0.01^{c}
16	α-Terpinyl acetate	1348	1.34 ± 0.12^{d}	2.85 ± 0.23^{c}	1.13 ± 0.04^{d}	5.79 ± 0.33^{a}	2.34 ± 0.14^{cd}	2.86 ± 0.18^{c}	4.46 ± 1.10^{b}	3.26 ± 0.14^{bc}
17	Neryl acetate	1363	0.11 ± 0.06^{c}	0.14 ± 0.00^{c}	$0.69 \pm 0.01^{a\text{-}c}$	0.12 ± 0.00^{c}	0.81 ± 0.05^{ab}	0.10 ± 0.00^{c}	0.27 ± 0.08^{bc}	1.14 ± 0.56^{a}
18	Geranyl acetate	1380	0.45 ± 0.18^{d}	0.13 ± 0.01^{d}	2.99 ± 1.27^{c}	0.71 ± 0.05^{d}	10.83 ± 0.73^{a}	1.18 ± 0.38^{d}	5.81 ± 0.19^{b}	0.14 ± 0.01^{d}
19	Methyleugenol	1404	0.38 ± 0.14^{e}	0.19 ± 0.01^{e}	7.73 ± 0.19^{a}	1.00 ± 0.06^{d}	6.46 ± 0.19^{c}	0.10 ± 0.01^{e}	7.19 ± 0.02^{b}	0.10 ± 0.00^{e}
20	*trans*-Caryophyllene	1416	0.11 ± 0.00^{b}	0.12 ± 0.01^{b}	0.10 ± 0.01^{b}	0.05 ± 0.03^{b}	1.35 ± 0.14^{a}	0.11 ± 0.01^{b}	0.05 ± 0.03^{b}	0.12 ± 0.01^{b}
21	α-Humulene	1451	0.23 ± 0.06^{b}	0.13 ± 0.01^{cd}	-	0.18 ± 0.01^{bc}	0.60 ± 0.02^{a}	0.12 ± 0.01^{cd}	0.07 ± 0.04^{de}	0.12 ± 0.01^{cd}
22	Spathulenol	1583	0.11 ± 0.00^{b}	0.14 ± 0.02^{b}	-	0.10 ± 0.01^{bc}	0.31 ± 0.06^{a}	0.13 ± 0.00^{b}	0.12 ± 0.00^{b}	0.16 ± 0.03^{b}
23	Caryophyllene oxide	1609	0.12 ± 0.00^{b}	0.13 ± 0.01^{b}	0.10 ± 0.00^{b}	0.14 ± 0.02^{b}	0.43 ± 0.08^{a}	0.15 ± 0.02^{b}	0.11 ± 0.01^{b}	0.18 ± 0.01^{b}
	Total (%)		94.91	96.98	97.53	94.21	92.97	94.31	97.78	94.38

According to the Duncan test application: means of the same column and main variable labeled with the same letters are not significantly different at $p < 0.05$.

The amount of α-pinene differed in leaves from 8.88% after the fully mature fruit dispersal stage to 32.84% at the flowering stage; the low α-pinene after the fully mature fruit dispersal stage could be due to the fact that the leaves had become stiff as harvesting occurred during the winter season when temperatures were low. At the unripe fruit stage, however, the leaves were brittle, and the harvest corresponded with the early summer season when the temperatures were high. In another study, the greatest amount of α-pinene was acquired 30 days after flowering and the lowest amount 180 days after flowering [20]. In the study of Aidi Wannes et al. [26], the highest amount of α-pinene was observed in the leaves than in the flowers and the stems, which is consistent with the present study. Limonene, which is the dominant component at the flowering stage, was found to be 23.97% in the flowers; however, the amount of limonene was insignificant at other harvest stages. In a study presented by Aidi Wannes et al. [20], the greatest amount (10.11%) was procured in the flower organ, while 1,8-cineole was one of the most dominant components at all phenological stages of development.

The highest amount of this compound was obtained at the before-flowering stage at 45.98%. Given that EO production after the fully mature fruit dispersal stage was 44.54%, these two stages were not statistically different from each other. In comparison, the lowest amount was 12.56% at the flowering stage in the flower organ (Table 1). In this regard, other researchers reported the lowest amount of 1,8-cineole in the flower organ and the greatest amount in the stem [20]. In another study, the lowest amount of 1,8-cineole was achieved at 30 days after flowering, while the greatest amount was obtained at 60 days after flowering [26]. Boelens et al. [60] found that during ripening the concentration of the main constituents changed, e.g., 1,8-cineole increased from 19.5 to 61.5%.

Linalool was another significant compound, the greatest amount of which was obtained in the leaf organ at the ripe fruit stage and the lowest amount at the before-flowering stage. Other researchers also reported that the linalool production is increased at the inception of the fruit development stage and that the lowest amount of linalool is found in the leaves, in comparison to the other organs [20,26].

α-Terpineol was another important compound found in myrtle EO, the highest amount of which was obtained in the leaves at the unripe fruit stage, and the lowest amount was at the before-flowering stage (3.57%). In a study presented by Aidi Wannes et al. [26], the greatest amount of α-terpineol was obtained at 90 days after flowering, which was almost equal to the unripe fruit stage, while the lowest amount was obtained 30 days after flowering, which is compatible with the current research.

Methyl citronellate and geranyl acetate were the other important compounds and the greatest amounts of these were procured at the unripe fruit stage in the fruit, at 5.04 and 10.83%, respectively. An increase in the amounts of these two compounds as a result of fruit development was also reported by other researchers [26].

Several studies provide evidence to support the hypothesis that the effects of phenological stages on EOs and their composition are due to their impact on the enzymatic activity and metabolism associated with EO production. The ontogeny of plants is closely related to the accumulation of secondary metabolites in plants, which in turn can affect the composition of EOs. A plant of each age accumulates different amounts of bioactive molecules according to its internal capacity for organ development. The age of the plant determines the composition of bioactive molecules both qualitatively and quantitatively [50,61].

The relative values of the different classes of EO compounds in various samples are represented in Figure 3. Oxygenated monoterpene was the dominant component in terms of EO production, the greatest amount being 72.37% in the leaves after the fully mature fruit dispersal stage, followed by 71.83% in the leaves at the ripe fruit stage, while the lowest amount found was 38.21% at the flowering stage in the flower organ. 1,8-cineole and linalool were the dominant components in EO production, which increased the class of oxygenated monoterpene in these organs. Other researchers have also mentioned that the dominant components in the EO in different populations of myrtle are the oxygenated monoterpenes, with the dominant compounds being 1,8-cineole and linalool [1,10,26,62].

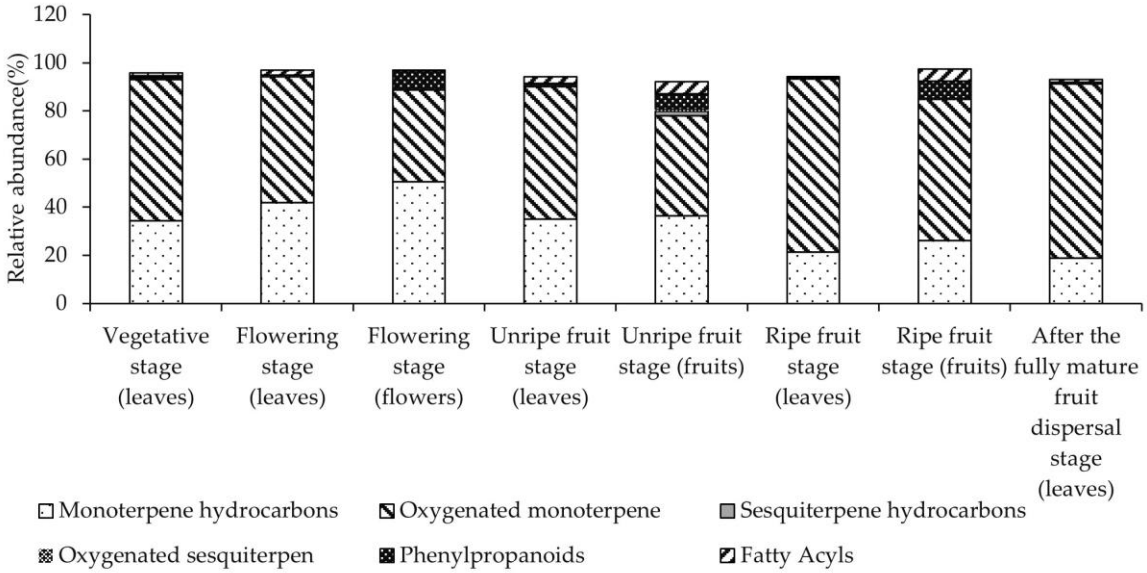

Figure 3. Comparison of main chemical groups (%) of myrtle organs in different phenological stages.

Monoterpene hydrocarbons were the second dominant compounds; the greatest amount being 50.52% at the flowering stage in the flower organ and the lowest amount 18.89% after the fully mature fruit dispersal stage in the leaves, of which the dominant compound was α-pinene. In the study of Aidi Wannes et al. in 2010, the dominant compound in the flower EO of myrtle was monoterpene hydrocarbons.

The quantity of monoterpene hydrocarbons decreased between the flowering and mature stages, while the amount of oxygenated monoterpene increased (Figure 3). Although monoterpenes are dominant in EOs, the distribution of oxygenated monoterpenes and monoterpene hydrocarbons varies. The dominant compounds producing EOs in myrtle were the monoterpenes, which were observed in the leaf organ more than the other organs at all the phenological stages, the lowest amount of which was seen at the unripe fruit stage in the fruit organ, while the greatest amount was seen at the flowering stage in the leaf organ. In other studies, the dominant compounds were reported to be the monoterpenes, which differed depending on oxygenated monoterpenes and monoterpene hydrocarbons population in the organs [1,26,62]. In the current study, the amount of sesquiterpene was very insignificant, which was also reported in the other studies. It should be noted that the quantity of EO compounds in plants has a direct relationship with biosynthesis, metabolism and biological activity of plants, which is a function of the climatic conditions of the environment [48].

3.3. Antioxidant Activity, Total Phenol and Flavonoid Contents

The results evidenced that there are significant differences in the amounts of phenol, flavonoid and antioxidant properties in the various organs of myrtle at the different phenological stages. This varied due to the use of the plant material collected during different phenological stages. The amount of antioxidant activity is shown in Table 2: With a low amount of the half maximal inhibitory concentration (IC_{50}), there was more antioxidant activity, and the before-flowering stage had the greatest antioxidant activity, equal to 31.04 μg/mL. The lowest activity was observed at the ripe fruit stage in the fruit organ and was equal to 835.68 μg/mL. These results demonstrated the high antioxidant properties of myrtle extract. In other studies, researchers also reported that the amount of antioxidant activity in different organs of myrtle is high [20,63]. Various reports have shown that the extract and the EO obtained from myrtle have high antioxidant properties [8,64]. The amount of antioxidant activity in the leaf organ at all the harvest stages was greater than in other organs in the current research, while the amount of antioxidant activity was reduced by the development of the flowers and fruits.

Table 2. Contents of total phenols and flavonoids and antioxidant activity of different myrtle organs in different phenological stages.

Traits	Vegetative Stage (Leaves)	Flowering Stage (Leaves)	Flowering Stage (Flowers)	Unripe Fruit Stage (Leaves)	Unripe Fruit Stage (Fruits)	Ripe Fruit Stage (Leaves)	Ripe Fruit Stage (Fruits)	After the Fully Mature Fruit Dispersal Stage (Leaves)
Total flavonoids content (mg QE/g DW)	7.49 ± 0.18^{a}	5.41 ± 0.29^{c}	3.68 ± 0.10^{e}	5.71 ± 0.15^{c}	4.79 ± 0.10^{d}	6.23 ± 0.15^{b}	2.69 ± 0.16^{f}	5.84 ± 0.09^{bc}
Total phenolic content (mg GAE/g DW)	66.52 ± 0.40^{a}	43.92 ± 0.05^{c}	27.50 ± 0.29^{e}	49.67 ± 0.77^{b}	18.83 ± 0.60^{f}	$30.22 \pm 1.75_{d-f}$	15.30 ± 0.75^{g}	32.33 ± 1.45^{d}
IC^{50}(μg/mL)	31.04 ± 0.54^{f}	273.30 ± 8.83^{e}	519.89 ± 20.84^{b}	355.26 ± 2.97^{d}	469.50 ± 1.04^{c}	476.17 ± 6.99^{c}	$835.68 \pm 18.23_{a}$	294.27 ± 14.45^{e}

According to the Duncan test application: means of the same column and main variable labeled with the same letters are not significantly different at $p < 0.05$. QE: quercetin equivalent; DW: dry weight; GAE: gallic acid equivalent; IC_{50}: half-maximal inhibitory concentration.

Compounds such as phenols and flavonoids, which are widely distributed in the different organs of myrtle, can enrich the plant's antioxidant properties. The increase in the antioxidant activities and the ability to purify the resulting free radicals make this plant useful for human health and explain why it is extensively utilized [65]. In the current experiment, the total phenol and flavonoid contents in various organ extracts were significantly different at the different phenological stages, which are presented in Table 2. In addition, we found in this study that biosynthesis and accumulation of phenol and flavonoids occur independently in each plant organ, so great variation in their synthesis is evident at different phenological stages. The flavonoid content at the flowering stage in leaves was higher than in the flowers and fruits at each stage. The greatest phenol and flavonoid contents were obtained at the before-flowering stage and were 66.52 mg GAE/g and 7.49 mg QE/g, respectively. The lowest amounts were obtained at the ripe fruit stage in the fruit organs, and they were 2.69 mg QE/g and 15.30 mg GAE/g, respectively. As the results show, the total phenol and flavonoid contents in the leaf organ were significantly greater than that in the flower and fruit organs at all phenological stages. Extract composition may significantly vary, depending on plant organ used for extraction [18,20,64,66,67]. Amensour et al. [65] demonstrated that leaf extracts contain significantly greater quantities of total phenolic compounds than mature fruit extracts. Flavonoids mainly accumulate in young plants, and their concentration is reduced after the flowering stage when the plant is actively differentiating rather than synthesizing metabolites [68]. An increased activity of the phenylalanine ammonia-lyase enzyme (PAL) in the vegetative and flowering stages may be responsible for the enhanced production of phenolic compounds [69]. Some multifunctional transcription factors and regulator proteins have been proposed to be responsible for the development-dependent accumulation of phenolic compounds in plant tissues [69]. In an experiment on *Sophora flavescens*, the expression pattern of the genes encoding enzymes involved in the phenylpropanoid pathway and some flavonoid pathway-specific enzyme isoforms were affected by the organs or phenological stage. The activity of PAL as a key enzyme of the phenylpropanoid pathway was enhanced by increasing the production of phenylpropanoids products and varied with the phenological stages. Different expression patterns of various copies of genes encoding the phenylpropanoid pathway enzymes (PAL, and cinnamate−4-hydroxylase) are related to physiological conditions and development. This might be one reason for the variable production of different phenolic compounds.

According to Maina et al. [70], low levels of phenolic compounds in plant young organs are connected with low quantities of lignin precursors in the cell wall, which is a result of low tissue lignification, which is contrary to our study's findings. Flavonoids are most concentrated in young plants and decrease during flowering the moment that the plant stops synthesizing metabolites [70]. In the study of Aidi Wannes et al. [26], the phenol and flavonoid contents in the leaf organ of myrtle were higher than those found in the flower and stem, which is compatible with the results of the current study. The

phenol and flavonoid contents were significantly reduced by increasing the number of the flowering days in myrtle, which is compatible with the current study [20]. As a result of the development and maturation of the fruit, a decrease in the phenol and flavonoid contents was also reported in other products [71,72]. During ripening, ethylene is released, which activates the transcription genes for the synthesis of various enzymes which degrade the phytoconstituents and are involved in the ripening process [73].

The high antioxidant properties in this plant occur due to the existence of the phenolic and flavonoid compounds in the extract. At the stage of the phenological and organ development where the greatest amount of the antioxidant activity was achieved, the highest total phenol and flavonoid contents were obtained at the same stage and from the same organ. As demonstrated in various studies, the antioxidant capacity of the herbal extracts is closely related to the phenolic contents [74–77]. When the concentration of phenolic compounds is high, the possibility of transferring hydrogen to free radicals is increased, and, consequently, the inhibitory power of the extract is increased [78]. Therefore, this phenomenon could lead to improvements in our understanding of the importance of phenolic compounds.

As part of the physiological process of plant ontogenesis, modifications to the secondary metabolism accompany morphological changes in plants. The phenolic content and antioxidant activities of plants in different phenological stages are likely to reflect major physiological and metabolic changes throughout their lifetime [79].

3.4. Correlation and Principal Component Analysis

An analysis of correlations was conducted to identify whether there was a relationship between phytochemicals and whether it was positive or negative. Based on the obtained results, it was shown that there was a positive and significant correlation between γ-terpinene with trans-caryophyllene (0.98), α-humulene (0.97) and caryophyllene oxide (0.95) (Table S1). A positive and significant correlation was also observed between the caryophyllene oxide with γ-terpinene (0.95), trans-caryophyllene (0.97), α-humulene (0.93) and spathulenol (0.91). On the other hand, a negative and significant correlation was observed between linalool and α-pinene (−0.86), δ−3-carene (−0.85) and p-cymene (−0.81). There is also a significant negative correlation between IC_{50} with β-pinene (−0.72), δ−3-carene (−0.79), flavonoids (−0.77) and total phenols (−0.87) (Table S1).

Many researchers have attempted to use correlation analysis in the plants of Salvia officinalis [80], *Trachyspermum ammi* [81], *Oliveria decumbens* [34], *Satureja hortensis* [82], *Satureja hortensis* [83], *Satureja rechingeri* [84], *Stachys Schtschegleevi Sosn* [85], *Heracleum persicum* [86] and *Satureja montana* [87] in order to find a relationship among the different phytochemical compositions.

The results of the principal component analysis based on all the recognized phytochemical compositions in the various organs of myrtle are represented in Table 3. According to the results obtained from the principal component analysis, it was evident that if the first five components have an eigenvalue greater than one, they have the greatest amount of relative variance, with the percentages of 35.64, 19.87, 16.19, 11.01 and 8.60%. In combination, they are responsible for 91.32% of the total variance. The results of the principal component analysis demonstrated that in the first component, isobutyl isobutyrate, β-pinene, δ−3-carene, p-cymene, geranyl acetate, methyleugenol, total phenols and IC_{50} obtained the highest factor loadings. Additionally, in the second component, it was seen that the compounds of γ-terpinene, trans-caryophyllene, α-humulene, spathulenol and caryophyllene oxide had the highest factor loadings. On the other hand, in the third component, the two compounds of α-thujene and limonene had the highest factor loadings. In the fourth and fifth components, the compounds of α-terpineol and the EO had the highest factor loadings.

Table 3. Principal component analysis of phytochemical compounds of myrtle organs in different phenological stages.

Compounds	PC1	PC2	PC3	PC4	PC5
Isobutyl isobutyrate (x_1)	−0.772	0.098	−0.339	−0.335	0.123
α-Thujene (x_2)	0.280	0.310	0.761	0.160	−0.190
α-Pinene (x_3)	−0.621	0.398	0.571	0.311	−0.082
β-Pinene (x_4)	−0.828	0.135	−0.040	−0.352	−0.106
δ−3-Carene (x_5)	−0.810	0.498	0.230	−0.172	0.035
p-Cymene (x_6)	−0.843	0.431	0.228	0.074	−0.198
Limonene (x_7)	0.137	−0.474	0.736	−0.009	0.411
1,8-Cineole (x_8)	−0.636	−0.029	−0.589	−0.455	−0.114
γ-Terpinene (x_9)	0.574	0.786	0.002	−0.114	0.138
Linalool (x_{10})	0.556	−0.507	−0.500	0.052	0.191
α-Terpineol (x_{11})	0.358	−0.234	−0.250	0.782	0.000
Methyl chavicol (x_{12})	−0.361	0.290	0.617	0.566	0.067
β-Citronellol (x_{13})	−0.500	0.363	−0.038	0.621	−0.056
Linalyl acetate (x_{14})	0.396	0.146	−0.676	0.511	0.024
Methyl citronellate (x_{15})	0.611	0.402	−0.055	−0.010	−0.572
α-Terpinyl acetate (x_{16})	0.161	−0.201	−0.521	0.515	−0.561
Neryl acetate (x_{17})	0.494	−0.036	−0.125	−0.283	0.668
Geranyl acetate (x_{18})	0.851	0.405	0.204	−0.208	−0.155
Methyleugenol (x_{19})	0.738	−0.134	0.581	−0.248	−0.161
trans-Caryophyllene (x_{20})	0.648	0.731	0.050	−0.044	0.195
α-Humulene (x_{21})	0.430	0.881	−0.120	−0.006	0.062
Spathulenol (x_{22})	0.442	0.777	−0.387	−0.050	0.040
Caryophyllene oxide (x_{23})	0.647	0.705	−0.157	0.009	0.243
Essential oil content (x_{24})	−0.165	−0.087	−0.061	0.547	0.751
Flavonoids (x_{25})	−0.622	0.435	−0.574	−0.034	0.020
Total phenols (x_{26})	−0.848	0.245	−0.016	0.142	−0.005
IC50 (x_{27})	0.721	−0.450	0.106	−0.054	−0.402
Eigenvalue	9.623	5.365	4.372	2.973	2.322
Relative variance (%)	35.641	19.871	16.194	11.012	8.602
Cumulative variance (%)	35.641	55.513	71.707	82.719	91.321

The biplot chart based on the first and second components represents the relationship of the different myrtle organs analyzed with the recognized phytochemical compositions (Figure 4). Therefore, the organs of vegetative stage (leaves) and flowering stage (leaves) were classified into one group based on the different phytochemical compositions, which had a stronger relationship with the compounds of 1,8-cineole, isobutyl isobutyrate, β-pinene, total phenols, p-cymene, δ−3-carene, α-pinene, flavonoids, β-citronellol and methyl chavicol. Furthermore, the organs of the flowering stage (flowers), unripe fruit stage (leaves), ripe fruit stage (leaves), ripe fruit stage (fruits) and after the fully mature fruit dispersal stage (leaves) were classified into one group based on the first and second components, which had a stronger relationship with the compounds of limonene, linalool, α-terpineol, α-terpinyl acetate, neryl acetate, methyleugenol, EO and IC_{50}. The biplot chart demonstrated that the unripe fruit stage (fruits) was classified into a distinct group because of its distance from the other two groups, which had a stronger relationship with the compounds of α-humulene, spathulenol, γ-terpinene, trans-caryophyllene, caryophyllene oxide, α-thujene, methyl citronella, geranyl acetate and linalyl acetate.

As one of the statistical methods for reducing data in biological and phytochemical studies, the principal component analysis methodology is useful for expressing a large quantity of data in a way that researchers can understand and therefore is widely used in these fields [88].

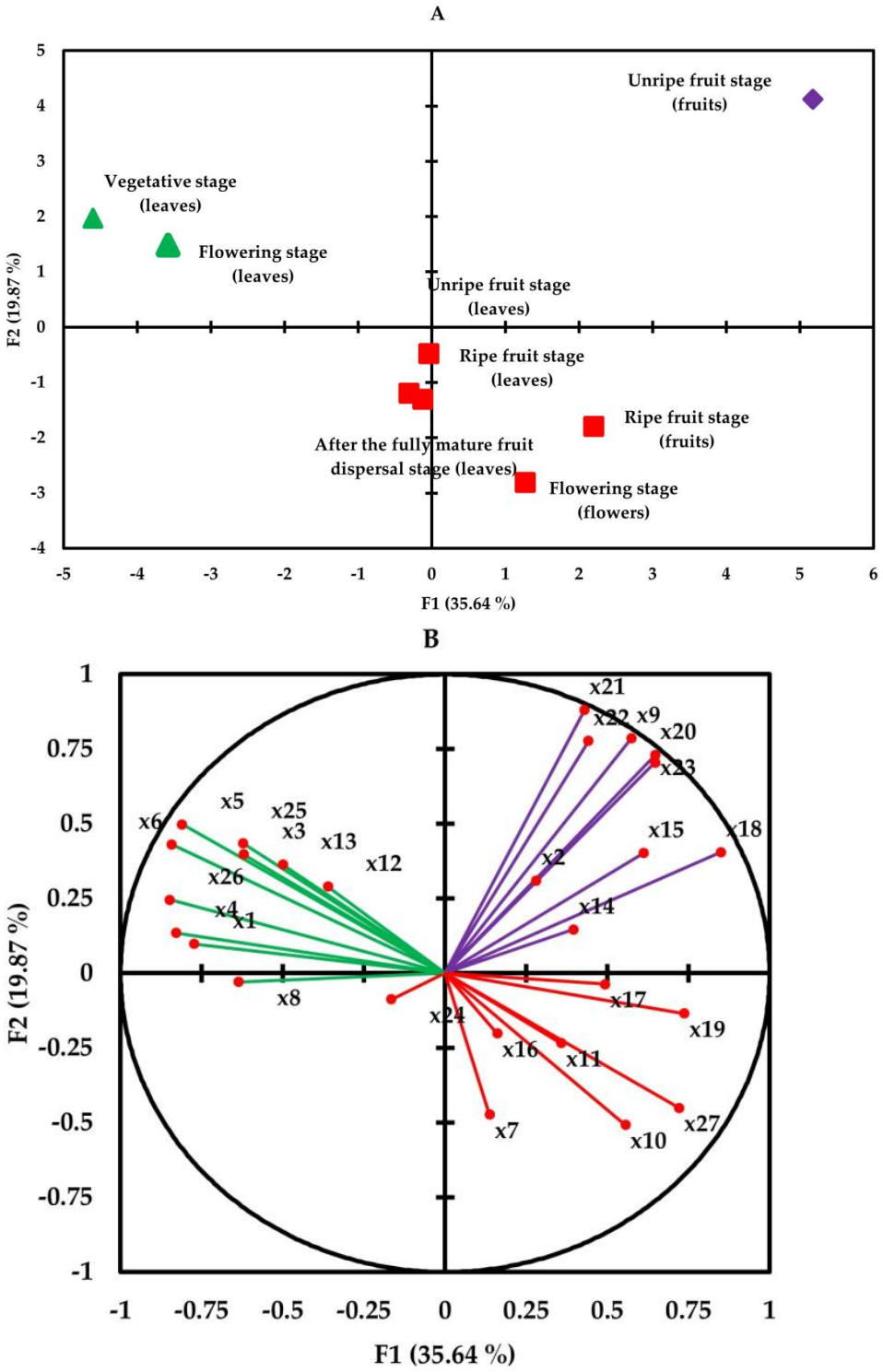

Figure 4. A: Biplot obtained from first and second components of myrtle organs based on different phenological stages (**A**) and phytochemical compounds (**B**).

4. Conclusions

This study reported the phytochemical analysis of the various organs of myrtle extracts collected at different phenological stages. The results show that there are significant variations in the phytochemical compounds of the various organs at the different harvest stages, and these variations can have effects on the antioxidant properties of myrtle plant. The greatest amount of EO in leaves was obtained at the flowering and after the fully mature fruit dispersal stage. Monoterpenes were the most dominant class of compounds in

all harvest stages and organs. α-pinene, 1,8-cineole, limonene and linalool were the most abundant components in all the organs at the different phenological stages. Certainly, the variation rate in the dominant components was considerable at different stages. Moreover, in the current experiment, the greatest contents of total phenols, flavonoids and antioxidant properties were achieved at the vegetative stage. According to the results obtained from the principal component analysis, the different organs at the different phenological stages were classified into three distinct groups, so that the members of each group had the closest relationship with each other from the point of all the phytochemical compositions, antioxidant properties and content of phenols and flavonoids. Thus, different organs at the ripe fruit stage (leaves), unripe fruit stage (leaves) and after the fully mature fruit dispersal stage (leaves), flowering stage (flowers) and ripe fruit stage (fruits) were classified into one group. Furthermore, leaves at the vegetative stage and flowering stage were classified into another group, while fruits at the unripe fruit stage were classified into one distinct group. The results of this study could serve as a guideline for growers to produce desirable metabolites with undeniable economic benefits to both the food and pharmaceutical industries. Collecting leaves of myrtle at the vegetative, flowering stages and after the fully mature fruit dispersal stage is imperative because, in general, the quantity of essential oil content and phytochemical compounds in these two stages and in the leaf organ is higher.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8090757/s1, Tables S1. Correlation between phytochemical compositions.

Author Contributions: Conceptualization, S.H.; methodology, S.H., S.J.H. and S.N.; validation, S.H. and S.N.; formal analysis, S.H. and S.J.H.; investigation, S.H., S.N. and M.-T.E.; resources, S.H.; data curation, S.H., S.J.H. and M.-T.E.; writing—original draft preparation, S.H. and S.J.H.; writing—review and editing, S.H., S.N. and M.-T.E.; visualization, S.N.; supervision, S.H.; project administration, S.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Azarbaijan Shahid Madani University, grant number 1401/1510.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The support of university of Azarbaijan Shahid Madani is greatly appreciated.

Conflicts of Interest: The authors declare no conflict of interest.

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