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Research Article

Comparative study of polyphenolic content and antioxidant capacity in fruits of *Arbutus unedo*, *A. andrachne* and their natural hybrid *A.× andrachnoides*

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Abstract

Fruits of the three *Arbutus* species native in the Eastern Mediterranean region, i.e. *Arbutus andrachne*, *A. unedo* and their natural hybrid *A.* × *andrachnoides*, collected from two different regions in Greece (Kalamos and Varympompi), were analysed comparatively in order to determine their phenolic profile and measure the antioxidant activity using two different single electron transfer-based assays, ferric reducing ability (FRAP) and 2,2-diphenylpicrylhydrazil radical scavenging capacity (DPPH). The pomological characteristics of the hybrid were similar to *A. andrachne*, while *A. unedo* had about 2.0-2.5 times larger fruits, with 20% more moisture being 175% heavier. All three species and their biotypes from the two areas had high phenolic content and antioxidant properties and were clearly pulled in different groups. Total flavanols scored a very strong correlation to antioxidant capacity. Highest total flavanols concentrations were found in *A. andrachne* fruits, while the hybrid had similar concentrations with *A. unedo*. In samples from Kalamos, concerning condensed tannins, total flavones, flavonols, phenolics and antioxidant capacity FRAP, as well as total anthocyanin and delphinidin 3-O-galactoside, the hybrid presented average values compared to the two parental species, while *A. andrachne* had the highest values. A strong correlation was found between ortho-diphenols and total soluble solids, as well as of total phenolics and flavonoids with total anthocyanins. The polyphenolic content and the antioxidant capacity varied in different biotypes. *A. andrachne* biotype from Kalamos showed the highest antioxidant capacity and anthocyanin potential. All *A. unedo* biotypes and *A.* × *andrachnoides* from Varympompi showed higher concentrations of ascorbic acid compared to the other biotypes.

Keywords: anthocyanins; eastern Mediterranean *Arbutus*; flavonoids; minor fruits; phenolics

Introduction

Fruits were always a significant part of human diet. The increasing food industries competition in nowadays led to the introduction of new fruiting species for cultivation, such as the temperate forest minor fruits, some of which have a relative wide scale of cultivation since the 15th century. Furthermore, temperate

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minor fruits have a wide gene pool and could be more adaptive to new environmental conditions due to climate change (Holubec *et al.*, 2015). The recent years there is an increasing number of research papers about the high impact of minor fruits derived from native plants around the world because of their richness in bioactive phytochemicals and phenolics with antioxidative properties (Soobrattee *et al.*, 2005; Bounous *et al.*, 2009). Plant phenolics have both protective effects attributed to vitamins and carotene and can be used in synthetic compounds to enrich food nutritional value (Pasqualone *et al.*, 2015). In addition, the use of synthetic antioxidants has been associated with possible toxic effects on human health and tend to be replaced by natural ones also found in minor fruits (Caleja *et al.*, 2017).

Native plants from the wild, i.e. representative xerophytic species of the Mediterranean basin, with both ornamental characteristics and nutraceutical properties are of high importance and they are introduced more and more in the urban and suburban landscape apart from being used in landscape restorations and reforestations (Papafotiou and Martini, 2016; Vlachou *et al.*, 2019). Ericaceae family, involves widespread members well known for their pharmaceutical effects (Pavlović *et al.,* 2009). *Arbutus* L., is a genus that includes evergreen, xerophytic, sclerophyll taxa of the Ericaceae family. Four species and two natural hybrids out of the twelve Arbutus species found worldwide are typical members of the Mediterranean vegetation, and two of these species *A. unedo* L. (strawberry tree) and *A. andrachne* L. (Greek strawberry tree) and their natural hybrid *A.* × *andrachnoides* Link. (Flora Europaea, 1996) are native in the eastern Mediterranean region including Greece. All three of these species are very attractive for ornamental use (Cervelli *et al.*, 2012; Bertsouklis and Papafotiou, 2013) and of high value in forestry for reforestations because of their ability for resprouting from the lignotuber after a fire (Konstantinidis *et al.*, 2006).

Fruits of *A. unedo* have nutritional value and economic importance (Celikel *et al.*, 2008) being used for the production of honey with a characteristic bitter taste, jams, beverages, spirits and other products in food industry (Soufleros *et al.*, 2005; Tuberoso *et al.*, 2010). A number of studies on *A. unedo* (Alarcao-E-Silva *et al.*, 2001; Maldini *et al.*, 2019) and less on *A. andrachne* (Tenuta *et al.*, 2018) fruits have shown that they are an excellent source of phenolic compounds, vitamin C (ascorbic acid) and fat-soluble antioxidants, dietary fiber being poor in lipids and Na (Mosele *et al.*, 2011) and of higher values than other red small fruits (Guerreiro *et al.*, 2013). The antioxidant content of *A. unedo* is rated highly between 28 common fruits studied (Garcia-Alonso *et al.*, 2004) and is characterized by a 'sui-generis' sweet tart flavour due to high sugar and acid content making this suitable for different food applications (Alarcao-E-Silva *et al.*, 2001). Most plant parts of *A. unedo* have been used in folk medicine (Bonvehi *et al.*, 1994; González *et al.*, 2010; Sansanelli *et al.*, 2017; Morgado *et al.*, 2018) with promising results for industrial use (Salem *et al.* 2018). In the NW Turkey five strawberry tree genotypes were selected for extensive cultivation (Celikel *et al.*, 2008). *A. andrachne* has also been employed in folk medicine and there are reports on its constituents (Sakar *et al.*, 1991).

Thus, although both *A. unedo* and *A. andrachne* have been studied for their industrial value (Issa *et al.*, 2008; Barros *et al.*, 2010; Afifi-Yazar *et al.*, 2011; Mendes *et al.*, 2011; Miguel *et al.*, 2014; Abidi *et al.*, 2015), there are no data about the nutritional and phytochemical characteristics for use in the food and medicinal industry of their hybrid *A.* × *andrachnoides*. In a previous work, it was found greater phenotypic resemblance and genetic similarity of *A.* × *andrachnoides* with *A. andrachne* than with *A. unedo*. We found intriguing to see if this pattern will apply also to nutritional and phytochemical characteristics of the three species. In addition to the scientific interest, research on this issue coincides with the encouragement of the Food and Agriculture Organization for the use of underutilized species by enhancing the exploitation of their nutritional and pharmaceutical properties (FAO, 2010). The existence of *in vitro* propagation protocols and phylogenetic studies (Papafotiou *et al.*, 2013) that could reveal the discrimination of suitable genotypes of these three Arbutus species will further facilitate their introduction to the pharmaceutical and food industry.

The present research aims to study comparatively the fruits of the three natives in the Eastern Mediterranean region *Arbutus* spp., i.e. *A. andrachne*, *A. unedo* and their natural hybrid *A.* × *andrachnoides,* collected from two different areas, in terms of phenolic content and antioxidant activity, in order to estimate them as sources of nutritional and pharmaceutical components.

Materials and Methods

Sampling area and sample plant selection

Arbutus unedo, *A. andrachne* and *A.* × *andrachnoides* fruits were collected from two typical macchia forests located in the prefecture of Attica, Greece: Kalamos (450 alt., lat. 38°16'22.1"N, long. 23°52'13.3"E) and Varympompi (400 m alt., lat. 38°08'13.6"N, long. 23°47'41.0"E). At each forest, fruits from three shrubs of each Arbutus species were collected (i.e. total of six different populations) (Table 1). Morphological characters were used for plant identification. Shrubs of similar size (2-3 m in height and diameter) at least 100 m apart from each other were selected. Each sample contained 500-600 g of randomly collected fruits from each individual plant. Each sample was considered as one replication. The fruits were collected fully ripened, based on the fruit colour (Herrera, 1998). *A. unedo* and *A. andrachne* fruits were collected in December 2017 and *A.* × *andrachnoides* fruits were collected in January 2018. The fruits were collected in the morning, packed in plastic bags, carried to the laboratory in a portable refrigerator and stored at -80 °C. Measurements included the determination of fruit pomological characteristics, soluble solids, pH, total titratable acidity, total phenols, total anthocyanins and total carotenoids, as well as the determination of individual profiles in anthocyanins and organic acids.

Table 1. Biotype code of Arbutus species and sampling area

Biotype	Species	Area	Biotype code
1	*A. andrachne*	Varympompi	A-V
2	*A. andrachne*	Kalamos	A-K
3	*A.* × *andrachnoides*	Varympompi	H-V
4	*A.* × *andrachnoides*	Kalamos	H-K
5	*A. unedo*	Varympompi	U-V
6	*A. unedo*	Kalamos	U-K

Fruit pomological characteristics

As soon as fruits were taken to the laboratory the weight of each fruit (Mettler Toledo AB104-S/FACT Analytical Balance), as well as its length and width (largest diameter vertical to length) was recorded (with a digital pachymeter). Fruit moisture was calculated as the difference between fruit fresh and dry weight (fruits were dried for five days at 70 °C).

Reagents

The identification of different anthocyanin compounds took place according to the order of elution and the retention times of pure compounds. There were used anthocyanins (cyanidinc-3-O-glucoside, delphinidin-3-O-galactoside, peonidin-3-Oglucoside) of Extrasynthèse (Gemay, France). Organic acid standards (quinic, malic, ascorbic, citric and fumaric acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

For the fruit phenolic extraction, approximately 100 frozen fruits were manually homogenized. Afterwards, 1 g fresh homogenized tissue was mixed for two times with 4 mL extraction medium water/methanol/acetone/HCL (19/40/40/1), homogenized for 1 min at 8,000 rpm in an Ultra Turrax homogenizer and the extracts were shaken in controlled temperature vacuum at 150 rpm for 30 min at room temperature (25 °C) and one more time with 4 mL extraction medium methanol/water/HCL (80/19/1) to prepare the extracts. The supernatants were collected after centrifugation of the samples at 4,500 rpm for 10 min. The extraction procedure was repeated two more times; the fractions were combined and the supernatants were stored at -80 °C until analysis.

Soluble solids, pH and total titratable acidity

For the determination of fruit juice soluble solids ATAGO N1-a refractometer with a 0-32 °Brix measurement range with 0.28 °Brix increments were used (no temperature compensation). Total titratable acidity was measured by titration with 0.1 N NaOH solution and was expressed as citric acid. For the measurement of soluble solids and total acidity, 3.5 g homogenized tissue of fresh fruits were mixed with 10.5 ml deionized water and then the samples were centrifuged at 4,500 rpm for 10 min (Mitcham *et al.*, 1996).

Total phenols and anthocyanins

Somers and Evans, (1977) method with some modifications was used for the measurement of total phenols and anthocyanins. An extract-aliquot was dissolved in methanol 1:10. 0.5 mL of the dissolved extract was added to 10 mL HCL 1M in order to measure anthocyanins and total phenols. Tubes kept in the dark for 3 h and afterwards the concentration both of total anthocyanins and phenols was determined spectrophotometrically at 520 and 280 nm. Known solutions of catechin and malvidin 3-O glucoside (12.5-200 μg mL^{-1}) were used to construct a calibration curve to estimate the concentration of total phenols and total anthocyanins respectively and the data are expressed as mean (milligrams of catechin equivalent per gram of fresh matter).

Total flavonoid content

The colorimetric method (Dewanto *et al.*, 2002) was used for the total flavonoid content determination as following. At first an aliquot of the extract was dissolved in methanol 1:10 and afterwards 0.25 mL of the dissolved extract or catechin standard solution was mixed with 1.25 mL of distilled water in a test tube followed by addition of 75 μL of 5% $NaNO_2$ solution. 6 min later, 150 μL of a 10% $AlCl_3$. $6H_2O$ solution was added to stand for another 5 min followed by addition of 0.5 mL of 1 ml NaOH. The mixture was filled with distilled water to 2.5 mL, mixed well and absorbance was measured immediately against the blank at 510 nm. Known solutions of catechin (12.5-200 μg mL^{-1}) were used to construct a calibration curve to estimate the concentration of total flavonoids and the data are expressed as mean (milligrams of catechin equivalent per gram of fresh matter).

Total flavanols, flavones and flavonols

The dimethylaminocinnamaldehyde (DMACA) method (Vivas *et al.*, 1994) was used for the estimation of the total flavanol content. Extract (0.2 mL), diluted 1:40 with methanol, was added into a 15-mL tube and then 1 mL of DMACA solution (0.1% in 1 N HCl in MeOH) was added. The mixture was vortexed, allowed to react at room temperature for 10 min and the absorbance at 640 nm was read against the blank. Known solutions of catechin (12.5-200 μg mL^{-1}) were used to construct a calibration curve to estimate the concentration of total flavanols and the data are expressed as mean (milligrams of catechin equivalent per gram of fresh matter).

For the estimation of total flavones and flavonols, an aliquot of 2 mL of the test solution, 20 mL methanol and 1 mL 5% aluminium chloride in methanol (w v^{-1}) were mixed in a volumetric flask, the volume was filed with methanol up to 50 mL and the mixture was left for 30 min. The absorbance was measured at 425 nm (Bonvehi *et al.*, 1994).

Total condensed tannins content

Total tannins content was determined using a colorimetric method of methyl cellulose. An aliquot of the extract was dissolved in methanol 1:10 (Sarneckis *et al.*, 2006).

Treatment Sample: To a 10 mL centrifuge tube, 3.00 mL of methyl cellulose solution was added to the required volume of tannin-containing solution (0.25 mL of supernatant), and the tube was capped, inverted several times, and allowed to stand for 2-3 min. Following the addition of 2.00 mL of saturated ammonium

sulphate solution, the sample was made up to final volume with deionized water, mixed again, and allowed to stand for 10 min at room temperature before centrifugation, which was performed at 4,000 rpm for 5 min.

Control Sample: The same volume of tannin-containing solution was added as per the treatment sample. Following the addition of 2.00 mL of saturated ammonium sulphate solution, the sample was made up to final volume with deionized water (in place of the methyl cellulose polymer solution), mixed, and allowed to stand for 10 min at room temperature before centrifugation as above.

The concentration of total tannins was estimated from a calibration curve, constructed by plotting known solutions of catechin (12.5-200 μg mL^{-1}). The results are expressed as mean (milligrams of catechin equivalent per gram of fresh matter).

Total carotenoids and chlorophyll-a analysis

The protocol of Nagata and Yamashita (1992) was used for the estimation of carotenoids and chlorophyll-a. Samples of pigments were extracted with acetone-hexane (4:6). 1 g of fresh tissue and 4 mL extraction solution were mixed with vortex for 1 min. Samples were centrifuged at 5,000 rpm for 5 min. The supernatant was collected and the extraction procedure was repeated six more times. All fractions were combined. Then the optical density of the supernatant at 663 nm, 645 nm, 505 nm and 453 nm were measured by spectrophotometer at the same time. From this value, the content of chlorophyll-a and carotene was estimated using our proposed equations: Chlorophyl-a (mg 100 mL^{-1}) = $0.999A_{663}$-$0.0989A_{645}$. β-Carotene (mg 100 mL^{-1}) = $0.216A_{663}$-$1.22A_{645}$-$0.304A_{505}$ +$0.452A_{453}$.

Analysis by HPLC

Individual anthocyanins were analysed by the high-performance liquid chromatography (HPLC) method and the identification was based on comparing retention times and on-line spectral data in comparison with original standards. The calibration curves of each standard compound were used for the quantification stage. Calibration curves made by plotting known solutions (1.25-20 μg mL^{-1}) were used for the estimation of concentration. The results were expressed as mean (catechin mg equivalent per gram of dry matter). The analyses were performed using an HPLC Shimadzu Nexera comprising a gradient pump, Shimadzu Nexera X2, a ProStar model 410 AutoSampler and a ProStar model 330 Photodiode Array Detector on a reversed-phase Waters C18 x select (250 mm × 4.6 mm, 5 mm) column at a temperature of 25 °C.

To measure anthocyanins by HPLC, 1 mL of the supernatant was evaporated into a sample concentrator, at room temperature, under a stream of nitrogen gas and the pellet was dissolved in 20 mL of 50% methanol in water.

The composition of the eluent was H_2O/HCOOH (90:10, A) and CH_3OH (100, B) with the flow rate at 1 mL min^{-1}. The linear gradient program employed for the elution was: 5% B for 0 min, from 5% to 50% B in 25 min, from 50% to 95% B in 30 min, followed by a return to the initial conditions in 10 min and re-equilibration of the column. The chromatogram was monitored at 520 nm.

To separate the organic acids (quinic, malic, ascorbic, citric and fumaric acid), homogenized tissue was diluted 1:4 in 5% meta-phosphoric acid and the solution was membrane-filtered (0.22 μm). A 250 × 4.6 mm ID, 5 μm, Waters × select C18 column operating at 20 °C, under isocratic conditions (mobile phase flow rate 0.8 mL min^{-1}) was used for the HPLC analysis. The mobile phase was acidified with 0.1% formic acid in water solution and extracted acids were detected at 210 nm with a PDA (Photodiode Array) detector. Calibration curves were constructed by standard solutions (quinic, malic, ascorbic, citric and fumaric acid) at a range of acid concentration from 31.25-1,000 ppm, for the quantification of acids.

Statistical analysis

The results were expressed as mean ± SE (Standard Error) of the three replications (50 fruits each) and all determinations were analysed three times. Data were processed by analysis of Variance (ANOVA), and statistical significance was processed using the Student's test at $P < 0.05$. Statistical analysis and correlations

were obtained using JMP v. 11 statistical software (SAS Institute Inc., Cary, NC, USA). Principal Component analysis (PCA) was used in evaluating the measurements and their contribution to the variability of the biotypes studied (*Biniari et al.*, 2020).

Results

Fruit pomological characteristics

The fruits of *A.* × *andrachnoides* were red-orange, spherical, with slightly granular surface like *A. andrachne* as opposed to the granular surface of *A. unedo*, but their taste was sweet, like *A. unedo*, while *A. andrachne* fruits were rather astringent. In terms of length, width and weight the hybrid fruits had intermediate characteristics compared to the parental species (in Kalamos) or closer to *A. andrachne* (in Varympompi), while fruit moisture was similar to *A. andrachne* in both areas and 10-15% lower than *A. unedo* (Table 2), which had the largest fruits.

Table 2. Fruit pomological characteristics of *A. andrachne, A.× andrachnoides* and *A. unedo* from two different areas

Trait	A-K	H-K	U-K	A-V	H-V	U-V
Moisture (%)	51.87 ± 0.71 c	53.30 ± 1.82 c	67.81 ± 0.48 a	53.70 ± 0.98 c	52.64 ± 1.11 c	64.46 ± 0.37 b
Length (mm)	11.32 ± 0.38 e	14.58 ± 0.38 c	19.80 ± 1.08 a	14.33 ± 0.41cd	13.21 ± 1.06 d	17.67 ± 0.76 b
Width (mm)	11.70 ± 0.81 d	14.90 ± 0.80 b	21.21 ± 1.48 a	12.25 ± 0.26 cd	13.90 ± 0.98 bc	19.63 ± 1.04 a
Weight (g)	1.07 ± 0.07 d	1.87 ± 0.05 c	4.37 ± 0.14 a	1.15 ± 0.05 d	1.23 ± 0.07 d	2.95 ± 0.67 b

Values are the mean (± SE) of three analyses from three different lots of fruits. Values followed by different lowercase letter within each trait are significantly different at the 5% level, determined by the one-way Anova (Student's *t* test, $P < 0.05$).

Soluble solids, pH and total titratable acidity

In Kalamos, the hybrid and *A. andrachne* fruits showed the highest concentration of total soluble solids (27.20 and 28.00 °Brix) and *A. unedo* the lowest (20.40 °Brix), but this result was not repeated in Varympompi where all species had similar measurements (Table 3). PH values of all three species were low and showed stability. The highest value of pH (3.85 and 3.87) was recorded in A-K and A-V, and of total acidity in the H-K (0.77) and U-V (0.79) (Table 3). PH was strongly correlated with both citric and fumaric acid (Figure 1).

Phenolics and antioxidant capacity

A-K fruits had half the concentration of total flavonoids (20.51 mg catechin/g fresh tissue) compared to all other samples ranging from 54.82-56.83, but this biotype had the highest concentrations of ortho-diphenols, total flavanols, total flavones, total phenolics, condensed tannins and total antioxidant activity FRAP and DPPH (Table 4). Total-flavanols and condensed tannins did not differ in the two biotypes of all three species. In Kalamos the hybrid had average concentrations of ortho-diphenols, total-flavanols, total flavones and flavonols, total phenolics, condensed tannins, and total antioxidant activity FRAP and DPPH compared to the parental species, while in Varympompi this could only be seen for total-flavanols, condensed tannins and antioxidant activity DPPH (Table 4).

Table 3. Pulp characters

Trait	A-K	H-K	U-K	A-V	H-V	U-V
Total soluble solids (° Brix)	27.20 ± 0.46 a	28.00 ± 0.46 a	20.40 ± 0.23 c	24.26 ± 0.26 b	23.73 ± 0.26 b	24.80 ± 0.46 b
PH	3.85 ± 0.01 a	3.39 ± 0.01 d	3.45 ± 0.01 c	3.87 ± 0.01 a	3.71 ± 0.03 b	3.34 ± 0.01 e
Total acidity (g/citric acid/L fruit juice)	0.54 ± 0.02 c	0.77 ± 0.02 a	0.58 ± 0.02 bc	0.61 ± 0.01 b	0.53 ± 0.03 c	0.79 ± 0.02 a

Values are the mean (± SE) of three analyses from three different lots of fruits. Values followed by different lowercase letter within each trait are significantly different at the 5% level, determined by the one-way Anova (Student's *t* test, $P < 0.05$).

Table 4. Flavonoids, total condensed tannins and phenolics

Trait	A-K	H-K	U-K	A-V	H-V	U-V
Total flavonoids†	20.51 ± 1.72 b	54.82 ± 0.58 a	54.40 ± 0.32 a	56.83 ± 0.35 a	54.98 ± 0.50 a	55.98 ± 0.42 a
Ortho-diphenols††	1.59 ± 0.14 a	1.36 ± 0.08 ab	0.76 ± 0.04 d	1.11 ± 0.03 bc	0.98 ± 0.05 cd	1.12 ± 0.08 bc
Total flavanols†	4.06 ± 0.43 a	2.00 ± 0.03 c	1.78 ± 0.21 c	3.38 ± 0.29 ab	2.00 ± 0.32 c	1.78 ± 0.33 c
Total condensed tannins†	58.27 ± 2.70 a	51.61 ± 2.89 ab	44.15 ± 1.62 bc	51.68 ± 3.74 ab	49.88 ± 0.73 b	37.40 ± 2.04 c
Total flavones/ flavonols†††	0.64 ± 0.03 a	0.52 ± 0.03 ab	0.37 ± 0.03 c	0.44 ± 0.05 bc	0.54 ± 0.04 ab	0.41 ± 0.02 c
Total phenolics†	60.16 ± 1.16 a	47.64 ± 2.93 b	26.80 ± 3.05 c	18.80 ± 0.74 de	18.13 ± 0.75 e	24.35 ± 1.33 cd
Total antioxidant activity FRAP††††	36.09 ± 0.03 a	27.39 ± 0.26 abc	21.08 ± 2.33 c	30.14 ± 0.92 ab	24.11 ± 0.76 bc	26.08 ± 2.47 bc
Antioxidant activity DPPH††††	38.81 ± 1.04 a	28.97 ± 1.09 cd	28.52 ± 0.65 d	33.71 ± 1.14 b	33.32 ± 0.56 b	31.74 ± 1.14 bc

Values are the mean (± SE) of three analyses from three different lots of fruits. Values followed by different lowercase letter within each trait are significantly different at the 5% level, determined by the one-way Anova (Student's *t* test, $P < 0.05$). †mg catechin/g fresh tissue, ††mg caffeic acid/g fresh tissue, †††mg rutin/g fresh tissue, ††††mg trolox/g fresh tissue

Organic acids, anthocyanins, chlorophyll and carotenoids

There was variation in the organic acids content in different biotypes (Figure 2). The hybrid, compared to the parental species, presented average concentration of citric acid in both areas. The highest concentration of quinic acid was found in *A. unedo* in Varympompi, and the highest concentration of fumaric acid in *A. andrachne* in Kalamos (Table 5). A-V presented the highest concentration of malic acid (2,221.9 μg/g fresh tissue), over double from U-K (1,013.3 μg/g fresh tissue) that presented the lowest concentration. *A. andrachne* and the hybrid seemed to have higher malic acid concentrations than *A. unedo. A. unedo* in both areas and the hybrid in Varympompi had a content of 798.3-858.1 μg/g ascorbic acid, significantly higher than the other three biotypes, representing a better source of vitamin C.

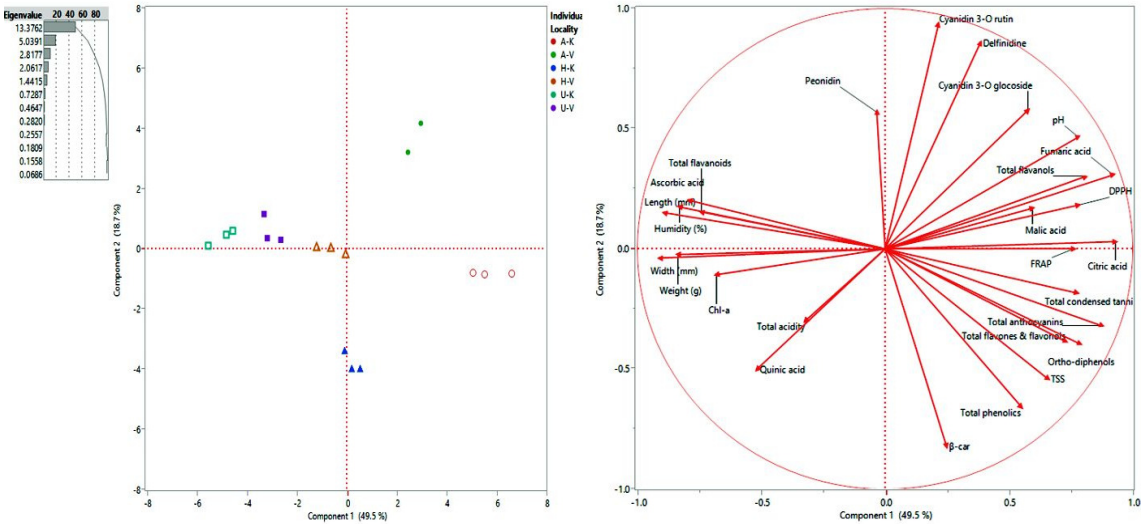

Figure 1. PCA analysis of the measurements studied and their contribution to the variability of the biotypes studied

The results of the anthocyanin profile of Arbutus biotypes revealed the presence of a total of four anthocyanins: delphinidin 3-O-galactoside, cyanidin 3-O-rutinoside, cyanidin 3-O-glucoside and peonidin 3-O-glucoside (Figure 3). The content of peonidin 3-O-glucoside had no difference between the different species and biotypes (Table 6) being the least abundant (0.03 to 0.11 mg/g fresh tissue). Total and individual anthocyanins concentrations did not seem to follow a pattern as far species and biotypes are concerned, apart from Kalamos where the hybrid exhibited average values of total anthocyanins and delphinidin 3-O-galactoside compared to the parental species (Table 6). The A-K exhibited the highest concentration of total anthocyanins (0.82 mg delphinidin 3-O- galactoside/g fresh tissue) and cyanidin 3-O-glucoside (0.53 mg/g fresh tissue) (Table 6). A-V presented the highest concentration of delphinidine 3-O-galactocide and cyanidin 3-O-rutinoside/cyaniding-3-O-glucoside equiv and the second highest cyanidin 3-O-glucoside (Table 6).

The concentration of total chlorophyll was very low in all biotypes, β-carotene was the only carotenoid detected and its highest concentration was recorded for H-K (0.037 mg/g fresh tissue, Table 5).

Table 5. Quinic, malic, ascorbic, citric, fumaric acid (Q-a, M-a, A-a, C-a, F-a respectively)

Trait[†]	A-K	H-K	U-K	A-V	H-V	U-V
Q-a	9,467.50 ± 801.80 b	10,912.30 ± 621.50 b	10,0445.30 ± 734.60 b	6,347.10 ± 105.20 c	10,439.60 ± 545.50 b	12,957.70 ± 469.00 a
M-a	1,511.20 ± 13.07 c	1,877.60 ± 13.46 b	1,013.30 ± 54.90 e	2,221.90 ± 47.96 a	1,291.71 ± 42.95 d	1,358.30 ± 30.91 b
A-a	498.70 ±11.49 b	512.30 ± 3.90b	841.80 ± 52.23 a	545.70 ± 2.08 b	858.10 ± 19.87 a	798.30 ± 9.42 a
C-a	5,056.20 ± 197.25 a	3,438.74 ± 34.94 c	2,815.36 ± 485.05 de	4,092.53 ± 119.99 b	3,196.91 ± 143.65 cd	2,639.97 ± 139.96 e
F-a	19.36 ± 0.92 a	9.89 ± 0.07 b	6.52 ± 0.08 c	17.47 ± 0.15 a	10.06 ± 0.46 b	10.25 ± 1.10 b

Values are the mean (± SE) of three analyses from three different lots of fruits. Values followed by different lowercase letter within each trait are significantly different at the 5% level, determined by the one-way Anova (Student's *t* test, $P < 0.05$). [†]$\mu g\ g^{-1}$ fresh tissue

Table 6. Total and individual anthocyanins, chlorophyll-a (chl-a), β-carotenoid (β-car)

Trait	A-K	H-K	U-K	A-V	H-V	U-V
Total anthocyanins†	0.820 ± 0.004 a	0.570 ± 0.023 b	0.370 ± 0.013 de	0.390 ± 0.004 d	0.500 ± 0.020 c	0.340 ± 0.010 e
Delphinidin 3-O-galactoside††	66.68 ± 5.04 b	41.10 ± 1.13 d	23.38 ± 5.13 e	126.83 ± 4.10 a	53.04 ± 3.26 c	74.64 ± 13.00 b
Cyanidin 3-O-rutinoside/cyanidin-3-O-glucoside equiv††	7.59 ± 0.26 c	3.89 ± 0.15 d	6.72 ± 0.24 c	12.76 ± 0.22 a	7.34 ± 0.26 c	9.11 ± 1.54 b
Cyanidin 3-O-glucoside††	0.53 ± 0.01 a	0.10 ± 0.01 f	0.15 ± 0.01 e	0.50 ± 0.01 b	0.27 ± 0.03 d	0.46 ± 0.03 c
Peonidin 3-O-glucoside††	0.04 ± 0.00 a	0.03 ± 0.00 a	0.08 ± 0.00a	0.16 ± 0.18 a	0.03 ± 0.00 a	0.11 ± 0.00 a
Chl-a†	0.0036 ± 0.0000 b	0.0039 ± 0.0000 b	0.0090 ± 0.0000 a	0.0008 ± 0.0000 b	0.0099 ± 0.0000 a	0.0088 ± 0.0000 a
β-car†	0.0190 ± 0.0000 b	0.0370 ± 0.0000 a	0.0100 ± 0.0000 d	0.0140 ± 0.0000 c	0.0210 ± 0.0000 b	0.0160 ± 0.0000 d

Values are the mean (± SE) of three analyses from three different lots of fruits.
Values followed by different lowercase letter within each trait are significantly different at the 5% level, determined by the one-way Anova (Student's *t* test, $P < 0.05$).
†mg delphinidin 3-O-galactoside /g fresh tissue
††mg/g fresh tissue

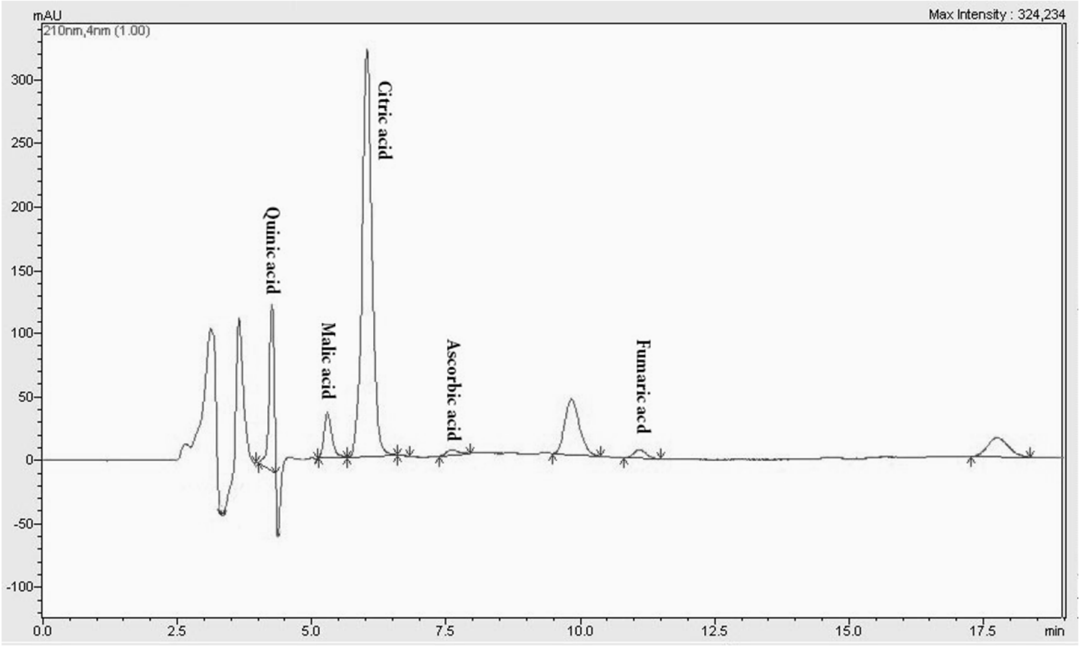

Figure 2. HPLC acid profile of *A. andrachne* (biotype A-K, from Kalamos) fruit extracts

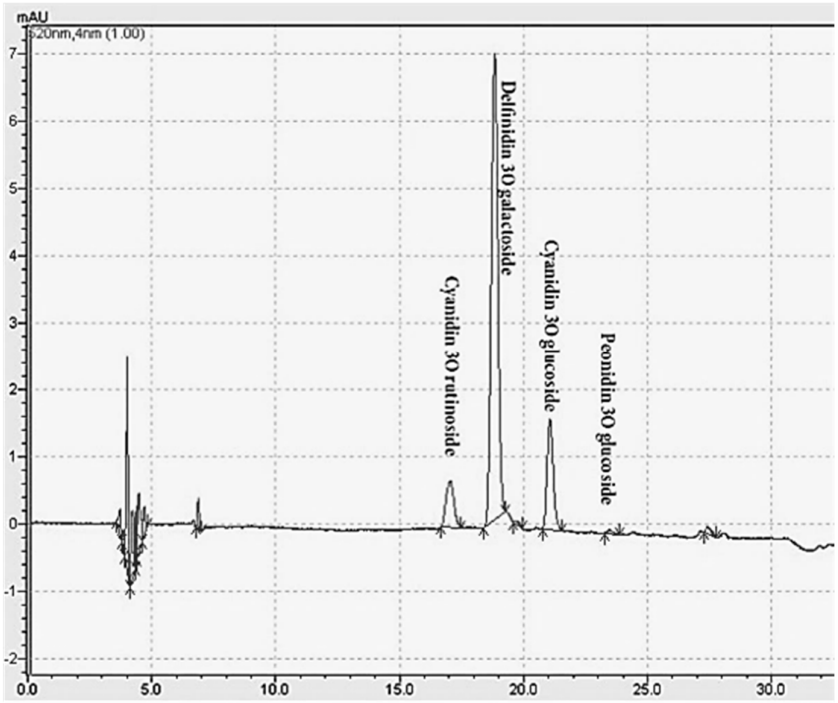

Figure 3. HPLC anthocyanic profile of *A. andrachne* (biotype A-V, from Varympompi) fruit extracts

Discussion

Fruit pomological characteristics

The morphology of the hybrid fruits was closer to that of *A. andrache* and only in the one area (Kalamos) some of the characteristics measured showed an average value between the two parental species. Average of two areas fruit mass of the hybrid was 1.55 g, closer to *A. andrachne* (1.11 g) and less than half of the *A. unedo* (3.65 g), which had the largest fruits with the highest moisture content (Table 2). Although the morphology of the hybrid fruits was closer to that of *A. andrachne* their taste was sweet, similar to *A. unedo*, while *A. andrachne* fruits were rather astringent due to their high concentration of tannins (Table 4).

The mass of *A. unedo* fruits was similar to that in two Spanish forests (Molina, *et al.*, 2011) and in Mersin Lapa-Gülnar N, Turkey (Ozcan and Haciseferogullari, 2007) and *A. andrachne* fruit mass was in accordance with Serce *et al.* (2010) data (1.15 g) in Turkey.

The hybrid fruits had similar moisture content to *A. andrachne*, while *A. unedo* fruits had the highest moisture, something expected because of their biggest size, as the main fruit components were shown to be moisture and carbohydrates (Ruiz-Rodríguez *et al.*, 2011). Moisture content in *A. unedo* fruits was of 64.46-67.81 (w/w), a bit higher to that counted by Özcan and Haciseferoğullari (2007) and Barros *et al.* (2010) (60 and 52% respectively) in fruits collected from N Turkey and Natural Park of Montesinho territory (NE Portugal) respectively. However, *A. unedo* fruit moisture was lower than the conventional fruits (75-95%), as shown for other wild fruits, e.g. *Vaccinium oxycoccus* (Souci *et al.*, 2008).

The geographic variation of fruit characteristics revealed by the data is referred also in previous studies of *A. unedo* fruit production (Herrera, 1998; Ogaya and Peñuelas, 2007; Molina *et al.*, 2011). It could be explained by variation of local soil characteristics and meteorological conditions that effects the extended reproductive cycle of all three species affecting also fruit production.

Soluble solids, pH and total titratable acidity

Total soluble solids and acidity in all three species were in the range found by Celikel *et al.* (2008) and Sulusoglu *et al.* (2011) for *A. unedo* in Turkish mountains and similar to other wild fruits, providing the unique flavor and making these fruits suitable in food technology. PH values of all three species were low (3.34 -3.85) and showed stability in agreement with previous studies on *A. unedo* (Cavaco *et al.*, 2007; Ruiz-Rodríguez *et al.*, 2011), but with remarkable difference with pH values (8.06 to 9.11) reported for eight different genotypes of *A. andrachne* by Serce *et al.* (2010). PH was strongly correlated with both citric and fumaric acid so that a simple measurement of pH could reveal biotypes with high concentration of citric or fumaric acid.

Phenolics and antioxidant capacity

In the area of Kalamos, *A. andrachne* fruits had the highest concentrations of ortho-diphenols, total flavanols, total flavones, total phenolics, condensed tannins, and total antioxidant activity FRAP and DPPH, followed by the fruits of the hybrid that had average values between the two parental species. Values of phenolic compounds and antioxidant properties falling between the parental species were also found in seeds but not in fruit skins of grape hybrids (Xu *et al.*, 2010), or in coconut oil from hybrids compared with their parents, where one hybrid was outstanding among the cultivars presenting the highest levels of lauric acid, total phenolic compounds, and antioxidant activity (Arlee *et al.*, 2013).

Ortho-diphenols is an important antioxidant polyphenolic group occurring in fruits, wine and olive, of high impact. Many authors have mentioned ortho-diphenol compounds as the most active antioxidants and of high antimicrobial activity (Bisignano *et al.*, 1999). In the present study, the biotype A-K had the highest concentration of ortho-diphenols with no difference from H-K (1.59 and 1.36 mg caffeic acid/g fresh tissue).

Antioxidant capacity is connected with anthocyanins, flavonoids and flavanols concentration of polyphenolic compounds (Garaguso and Nardini, 2016) and depends on their structure, the number and position of the hydroxyl groups and the nature of the substitutions on the aromatic rings (Balasundram *et al.*, 2006). The biological and nutritional value of fruits can be expressed by the determination of total antioxidant capacity with tests like DPPH (1,1-diphenyl-2picrylhydrazyl free radicals test) and FRAP (ferric reducing antioxidant power) (Koleva *et al.*, 2000; Gazdik *et al.*, 2008). It is difficult to compare the antioxidant capacity of the three Arbutus species with relevant reports in the literature due to different methods of determination used. However strawberry tree fruits ranked fourth between 28 different fruits (Garcia-Alonso *et al.*, 2004).

The values of phenolic content and antioxidant capacity of *A. andrachne* fruits confirmed previous data by Serçe *et al.* (2010) for this species in Turkey. There were also similarities between our results on *A. unedo* with previous studies on the phenolic profile of *A. unedo* samples from Italy, Portugal and Spain (Pallauf, *et al.*, 2008; Fortalezas *et al.*, 2010; Mendes *et al.*, 2011). Quantification of the comparison of phenolic compounds was not possible either due to the use of different units or lack of reporting the quantities in these studies.

Organic acids, anthocyanins, chlorophyll-a and carotenoids

A. unedo in both areas and the hybrid in Varympompi had a content of 498.7-858.1 μg g^{-1} ascorbic acid, significantly higher than the other three biotypes representing a better source of vitamin C, higher even than citrus fruits (Fatin and Azrina, 2017). Seker and Toplu (2010) who studied *A. andrachne* and *A. unedo* in Turkey, also found a higher concentration of ascorbic acid in *A. unedo* than *A. andrachne*, with mean values in both species much higher (3-4 times) than those found in the present study. On the other hand, in the present study all biotypes of the tree species had 9-15 times higher concentration of ascorbic acid in comparison to *A. unedo* fruits (55 μg g^{-1} fresh tissue) in Salamanca, West Spain (Pallauf *et al.*, 2008).

The anthocyanin profile of all Arbutus biotypes revealed the presence of four anthocyanins, i.e., delphinidin 3-O-galactoside, cyanidin 3-O-rutinoside, cyanidin 3-O-glucoside and peonidin 3-O-glucoside. In previous reports the anthocyanin profile of *A. unedo* was characterized by the existence of delphinidin and

cyanidin (Pallauf *et al.*, 2008) that has been identified as the characteristic red colour-factor of the fruit (Proloiac and Raynaud, 1981). In the present work *A. andrachne*, particularly in Varympompi, presented the highest concentration of delphinidin and cyanidin and indeed the colour of *A. andrachne* fruits are usually darker red than the *A. unedo* and the hybrid. The identification of delphinidin 3-O-galactoside, cyanidin 3-O-rutinoside and cyanidin 3-O-glucoside is similar with Pallauf *et al.* (2008) and Fortalezas *et al.* (2010) on *A. unedo* fruits study, although in the present study one more anthocyanin, peonidin 3-O-glucocide, is described in the anthocyanin profile of strawberry tree fruits. The concentration of A-V delphinidin 3-O-galactoside, reached up to 6 times higher than U-K concentration (126.83 and 23.38 mg g^{-1} fresh tissue respectively) and almost two times higher than U-V (74.64 mg/g fresh tissue), giving to A-V biotype a high impact because of the beneficial effects of anthocyanins both as antioxidants and regulators of adipocytokine gene expression and anti-cancer factors (Vattem and Shetty, 2005; Tsuda et *al.*, 2006).

The very low total chlorophyll content in fruits of all biotypes proved that they were collected at the full maturity stage. Regarding carotenoids analysis, β-carotene was the only carotenoid detected, in accordance to Alarcao-E-Silva *et al.* (2001) and Pallauf *et al.* (2008) reports for *A. unedo.*

Conclusions

The present study enriches the previous knowledge on the potential use of Arbutus species found in Eastern Mediterranean region, and for the first time provides information on the polyphenolic content and antioxidant capacity of the natural hybrid *A.* × *andrachnoides* in comparison with the two parental *species A. unedo* and *A. andrachne*. The hybrid fruits had pomological characteristics closer to that of the *A. andrache* in almost all biotypes, and values of ortho-diphenols, total flavanols, total flavones, total phenolics, condensed tannins, and total antioxidant activity FRAP and DPPH average between the two parental species.

The results obtained revealed the significant differences in the polyphenolic content among the various biotypes of the three species, whereas the *A. unedo* biotypes were closer to each other. Therefore, this study confirms earlier reports for a wide variability in the chemical composition and properties of *A. unedo* and *A. andrachne* fruits.

The antioxidant capacity positively correlates with the total flavanols, and the otho-diphenols with the total soluble solids. Four anthocyanidins were identified and quantified and *A. andrachne* biotype from Kalamos proved to have the highest antioxidant activity and anthocyanin potential. Both *A. unedo* biotypes and *A.* × *andrachnoides* from Varympompi had the highest concentration of ascorbic acid compared to the other biotypes, whereas *A. unedo* from Varympompi exhibited the highest concentration of quinic acid. With regards to the utilization of the species, the differences revealed within the present study in combination with previous phylogenetic, ecophysiology and *in vitro* propagation studies will allow both pharmacy and food industry to develop suitable strategies for the best utilization of those species.

Authors' Contributions

Conceptualization: KFB and KB; Data curation: KFB, ID and KB; Investigation: KFB and MP; Methodology: KFB, ID and KB; Validation: KFB; Resources: KB and MP; Supervision: MP; Writing - original draft: KFB, ID, KB and MP; Writing - review and editing: KFB and MP

All authors read and approved the final manuscript.

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Conflict of Interests

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

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