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Stability and antioxidant activity of polyphenols in extracts of *Myrtus communis* L. berries used for the preparation of myrtle liqueur

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Abstract

Flavonoids and anthocyanins in berry extracts from *Myrtus communis*, prepared by following a typical Sardinia myrtle liqueur recipe, were identified by HPLC coupled with Electrospray Mass Spectrometry and quantified by HPLC coupled with Ultraviolet/Visible Detection in order to evaluate the stability of the extracts during 1 year of storage. Antioxidant activity was measured by using TEAC assay, and the free-radical scavenging activity was monitored during time of the stability evaluation.

Anthocyanins have found to be the most instable compounds, but a considerable instability was observed also for flavonoids, suggesting the use of extracts not over 3 months from their preparation. The myrtle extract showed interesting free-adical scavenging activity. Antioxidant activity was preserved in 3 months.

Keywords: *Myrtus communis*; Anthocyanins; Flavonoids; Quantitative determination; Phenol stability; Free-adical scavenging activity

1. Introduction

Polyphenols constitute one of the most represented classes of compounds in higher plants including medicinal and edible plants (vegetables, fruits, etc.). In recent years, an increasing number of publications has been reported on the chemistry of flavonoids especially due to their biological properties (antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic, vasodilatory, anti-mutagenic and neoplastic) and their ability in the diet to protect against or inhibit the development of cancer [1,2].

Increased interest has been shown in exploring the benefits to human health due to the free-radical scavenging and antioxidant capacities of those compounds naturally available in fruits and vegetables [3]. Thus, flavonoids may enhance anti-inflammatory and estrogenic activities, inhibit lipid peroxidation and enzymes associated with tumour formation, provide protection from DNA cleavage, boost the production of cytokines, decrease capillary permeability and fragility, and maintain membrane integrity [4–6]. The antioxidant property of flavonoids has been the first mechanism of action studied, in particular with regard to their protective effect against cardiovascular diseases. Flavonoids have been shown to be highly effective scavengers of most types of oxidative molecules, including singlet oxygen and various free radicals [7] that are probably involved in several diseases.

Myrtus communis L. is a plant traditionally used as an antiseptic and disinfectant drug [8]. Concerning to the chemical study of the plant, several compounds have been isolated from the leaves [9,10], the essential oil [11] and the fruits [12]. The berry of *M. communis* L. is used to produce the characteristic myrtle liqueur typical of Sardinia, Italy. The fruit is spherical in shape, dark red to violet in colour, and is reported to contain delphinidin-, petunidin-, malvidin-, peonidin- and cyanidin-3-mono- and 3,5-diglucosides [12], along with glycosides of myricetin and quercetin. In a recent paper the arabinoside derivatives of delphinidin, malvidin, petunidin and cyanidin were identified in myrtle berries extracts [13].

The organoleptic properties of the liqueur are strongly associated with the phenolic content, and anthocyanidin show only

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limited stability [14,15]. For food applications, stability of anthocyanins is of great concern because they are usually little stable and sensitive to changes in pH. Consequently, many studies on the stability of anthocyanins have been published [16,17]. Factors affecting the colour and stability of anthocyanins include structure and concentration, pH, light, copigments, self-association, metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation products, proteins, sulphur dioxide, and the temperature and time of processing and storage conditions [18,19].

In addition *M. communis* berries can be collected only in a definite season, thus the myrtle liqueur production cannot be performed all year long and it is necessary to find a system for storing berries or their hydroalcoholic extracts. In order to know what happens to hydroalcoholic extracts during a definite store period, the evolution of phenolic constituents during the period of 1 year has been analysed.

In addition, for the first time free-radical scavenging activity of the extracts has been investigated by using the TEAC (Trolox equivalent antioxidant activity) assay [20], and the variability of this activity has been monitored in the time. A previous paper reports the antioxidant activity of myrtle liqueur [21].

Authors have previously reported an LC–MS method for the qualitative and quantitative determination of anthocyanins and anthocyanidins from myrtle berries extracts. In this technique, electrospray (ES) ionisation combined with an ion trap (IT) mass analyser was used together with low energy MS–MS for fragmentation studies [13]. In order to analyse simultaneously anthocyans and flavonoids a different LC method has been used here, and, considering that in some cases anthocyans and flavonoids have the same value of protonated molecular ions, UV detection has been preferred, in order to unequivocally distinguish the signal at 350 nm, specific for flavonoids and the signal at 520 nm, specific for anthocyans.

2. Experimental

2.1. Reagents and standards

HPLC grade methanol, acetonitrile and trifluoroacetic acid were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). HPLC grade water (18 mΩ) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. Standards of anthocyanins **1–5** and flavonoids **9–14** were purchased from Extrasynthese (Geney, France). Standards of anthocyanins **6–8** were isolated in our laboratories using the semi-preparative procedures described previously [13]. Standard stock solutions (1 mg/mL in methanol) of each of **1–14** were prepared. For compound identity see Fig. 1.

2.2. Preparation and analyses of myrtle extracts

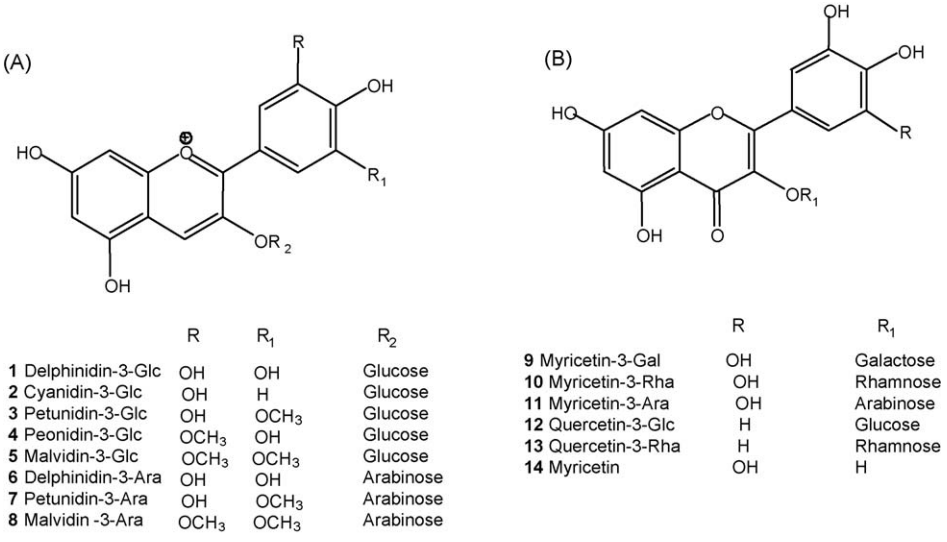
Berries of *M. communis* L. were collected in December 2002 in Sardinia, Italy and were supplied by Zedda Piras Company (Alghero (SS), Italy).

About 500 g of fresh berries were extracted according to the traditional recipe for the preparation of the liqueur consisting in macerating fresh berries (500 g) in ethanol:water (70:30; 960 mL) for 40 days.

About 500 g of fresh berries were lyophilized and then a sample of 130 g was extracted by macerating berries (130 g) in ethanol:water (70:30; 250 mL) for 40 days.

About 500 g of fresh berries were extracted by sonication enhanced maceration in ethanol:water (70:30; 960 mL), performing the extraction by sonication for 1 h followed by maceration for one night.

For quantitative determination, each extract was diluted 1:10 with ethanol:water 70:30, appropriate volumes of the internal standards (IS) cyanidin-3-*O*-galactopyranoside for anthocyans and rutine (quercetin-3-*O*-rutinoside) for flavonoids were added



	R	R_1	R_2
1 Delphinidin-3-Glc	OH	OH	Glucose
2 Cyanidin-3-Glc	OH	H	Glucose
3 Petunidin-3-Glc	OH	OCH_3	Glucose
4 Peonidin-3-Glc	OCH_3	OH	Glucose
5 Malvidin-3-Glc	OCH_3	OCH_3	Glucose
6 Delphinidin-3-Ara	OH	OH	Arabinose
7 Petunidin-3-Ara	OH	OCH_3	Arabinose
8 Malvidin -3-Ara	OCH_3	OCH_3	Arabinose

	R	R_1
9 Myricetin-3-Gal	OH	Galactose
10 Myricetin-3-Rha	OH	Rhamnose
11 Myricetin-3-Ara	OH	Arabinose
12 Quercetin-3-Glc	H	Glucose
13 Quercetin-3-Rha	H	Rhamnose
14 Myricetin	OH	H

Fig. 1. Phenolic constituents in *M. communis* berries. (A) Anthocyans. (B) Flavonoids.

to give a final concentration of 25 μg/mL, and a 20 μL aliquot injected into the analytical system: determinations were replicated five times each.

2.3. HPLC-UV/VIS analysis

An Agilent (Palo Alto, CA, USA) 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (20 μL injection loop), a G-1322A degasser and a G-1315A photodiode array detector was employed. Analyses were carried out using a Waters Simmetry C_{18} column (150 mm × 2.0 mm i.d.; particle size 5 μm) eluted with mixtures of 0.1% trifluoroacetic acid (TFA; solvent A) and acetonitrile:water (8:2) containing 0.1% TFA (solvent B) at a flow rate of 0.3 mL/min. Elution was by step gradient from 90:10 (A:B) to 80:20 in 15 min, then from 80:20 to 60:40 in 10 min, and subsequently from 60:40 to 40:60 in 10 min, from 40:60 to 30:70 in 10 min. Under these chromatographic conditions compounds **1**–**14** could be separated (see chromatograms in Fig. 2).

Detection was carried out with two wavelengths, 350 nm specific for flavonoids, and 520 nm, specific for anthocyanins.

2.4. LC–ES/MS

Qualitative on-line LC–ES/MS analyses of extracts were performed using the Thermo Finnigan Spectra System HPLC coupled with the LCQ Deca IT (Thermo Electron, San José, CA, USA) with the chromatographic conditions as described above. Electrospray ion source worked at the temperature of 280 °C, and the parameters were optimised for both the classes of compounds, and they were the followings: capillary voltage 38 kV, spray voltage 5 kV, tune lens offset −50. Nitrogen was supplied at the flow of 80 (arbitrary units).

The flow from the chromatograph was injected directly into the ES ion source and MS were acquired and elaborated using the software provided by the manufacturer. Reconstructed ion chromatograms were elaborated in order to identify flavonoids and anthocyanins by their protonated molecular ions.

2.5. Calibration and quantification

A sample (10 mg) of each flavonoid and anthocyanin standard was weighted accurately into a 10 mL volumetric flask, dissolved in methanol and the volume made up to the mark with methanol. The resulting stock solutions were diluted with methanol in order to obtain reference solutions containing 5, 25, 50 and 125 μg/mL, and to each reference standard solution was added an appropriate amount of IS to give a final concentration of 25 μg/mL. Calibration curves for each of the reference standards were constructed by injecting the standard solutions at each concentration level in triplicate. The ratios of the peak areas of the external standard (at each concentration) to those of the IS were calculated and plotted against the corresponding standard concentration using weighted linear regression to generate standard curves.

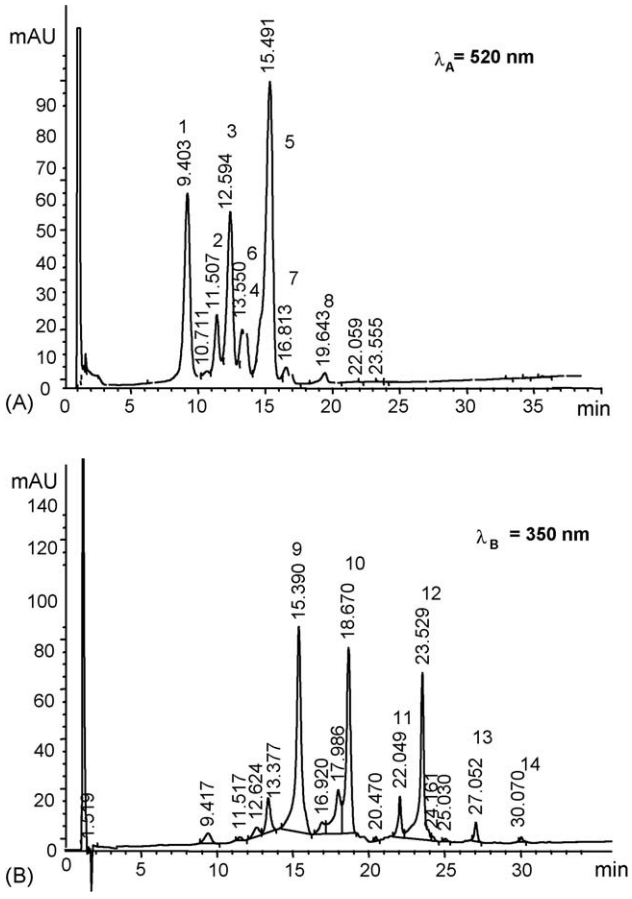


Fig. 2. HPLC-UV/Vis analysis of phenolic compounds in myrtle hydroalcoholic extracts. Panel A. Detection at 520 nm: **1**, delphinidin-3-*O*-glucoside; **2**, cyanidin-3-*O*-glucoside; **3**, petunidin-3-*O*-glucoside; **4**, peonidin-3-*O*-glucoside; **5**, malvidin-3-*O*-glucoside; **6**, delphinidin-3-*O*-arabinoside; **7**, petunidin-3-*O*-arabinoside; **8**, malvidin-3-*O*-arabinoside. Panel B. Detection at 350 nm: **9**, myricetin-3-*O*-galactoside; **10**, myricetin-3-*O*-rhamnoside; **11**, myricetin-3-*O*-arabinoside; **12**, quercetin-3-*O*-glucoside; **13**, quercetin-3-*O*-rhamnoside; **14**, myricetin. Chromatographic conditions: Stationary phase Waters Simmetry C_{18} column (150 mm × 2.0 mm i.d.; particle size 5 μm); mobile phase with 0.1% trifluoroacetic acid (TFA; solvent A) and acetonitrile:water (8:2) containing 0.1% TFA (solvent B). Elution was by step gradient from 90:10 (A:B) to 80:20 in 15 min, then from 80:20 to 60:40 in 10 min, and subsequently from 60:40 to 40:60 in 10 min, from 40:60 to 30:70 in 10 min. Flow rate 0.3 mL/min.

2.6. Stability test

A sample of 100 mL of the extract prepared by following the traditional recipe from fresh berries, was transferred in a dark bottle, and stored in the dark for 1 year. Monthly, a sample of 1 mL was taken, dried under nitrogen frozen and then stored. All the samples were analysed by reconstructing these samples in 1 mL of ethanol:water mixture, before the analysis.

2.7. TEAC assay

The in vitro antioxidant activities of hydroalcoholic extracts of the berries were determined by the Trolox equivalent antioxidant capacity (TEAC) assay [20] as previously reported [22,23]. The TEAC value is based on the ability of the antioxidant

to scavenge the radical cation $ABTS^+$ with a spectrophotometric analysis. The antioxidant activities of the hydroalcoholic extracts are expressed as TEAC values. The TEAC value is defined as the concentration of a standard Trolox (Sigma Chemical Co., St. Louis, MO) solution with the same antioxidant capacity as a 1 mM concentration of a tested compound. In the case of the extracts the TEAC value is defined as the concentration of a standard Trolox solution with the same antioxidant capacity as a 1 mg/mL of the tested extract.

3. Results and discussion

3.1. HPLC-ESI-MS and HPLC-UV/VIS of M. communis berries extracts

Myrtle berries fresh material was divided in three groups to submit to three different extraction procedures. About 500 g of fresh berries were extracted by maceration for 40 days using the traditional recipe for the preparation of the liqueur; 500 g of fresh berries were lyophilized and then a sample of 130 g was extracted by maceration for 40 days according to the traditional recipe; 500 g of fresh berries were submitted to extraction enhanced by ultrasonication, performing the extraction by sonication for 1 h followed by maceration for one night.

HPLC analysis of *M. communis* berry extracts with the analytical procedure described in Section 2 revealed the presence of eight anthocyans (**1**–**8**) (Fig. 1A), in the chromatographic profile obtained with detection at wavelength of 520 nm (Fig. 2A) and six flavonoids (**9**–**14**) (Fig. 1B) in the chromatographic profile obtained with revelation at wavelength of 350 nm (Fig. 2B), which had been previously isolated and identified.

All the 14 compounds were identified by a single run of LC-ESI-MS, by using the same chromatographic condition used during the HPLC-UV analysis, performing reconstructed ion chromatogram for the expected protonated molecular ions for flavonoids, and expected molecular ions for anthocyanins.

Major peaks in the anthocyanin profile corresponded to compounds **1**, **3** and **5**, respectively, identified as delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, and minor peaks were identified as compounds **2**, **4**, **6**, **7** and **8**, respectively, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, delphinidin-3-*O*-arabinoside, petunidin-3-*O*-arabinoside and malvidin-3-*O*-arabinoside.

Compound **4** (peonidin-3-*O*-glucoside) and **6** (delphinidin-3-*O*-arabinoside) were not well separated, thus they were quantified as a mixture. In addition, an anthocyanin previously identified by LC-ESI-MS was present in a very small amount (cyanidin-3-*O*-arabinoside), which prevented its quantification.

Major peaks in flavonoid profile corresponded to compounds **9**, **10** and **12**, respectively, identified as myricetin-3-*O*-galactoside, myricetin-3-*O*-rhamnoside and quercetin-3-*O*-glucoside. Minor peaks were identified as compounds **11**, **13** and **14**, respectively, myricetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside and myricetin.

A qualitative comparison of extracts obtained by maceration of fresh berries in 40 days (panel A), maceration of lyophilised berries in 40 days (panel B) and extraction enhanced by ultrasonic generator (panel C), was performed by LC-UV/VIS, and led to the results shown in Fig. 3.

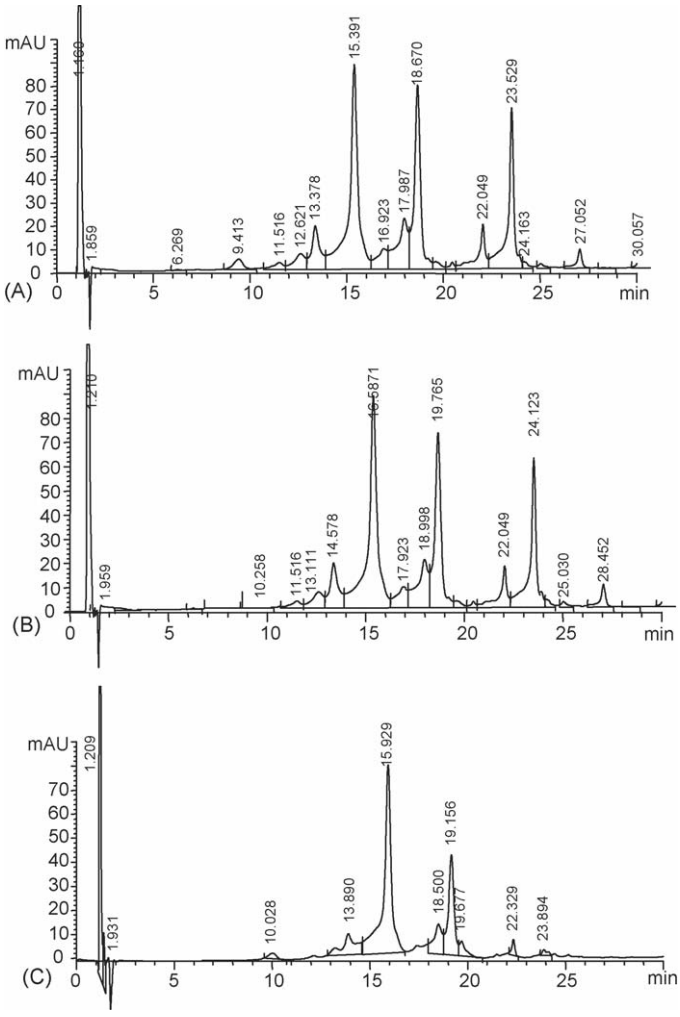


Fig. 3. HPLC-UV-vis (detection at 350 nm) chromatograms of (A). Fresh berries extracted by following the traditional recipe. (B) Lyophilised berries extracted by following traditional recipe. (C) Fresh berries extracted with ultrasonic generator. Chromatographic conditions: Stationary phase Waters Simmetry C_{18} column (150 mm × 2.0 mm i.d.; particle size 5 μm); mobile phase with 0.1% trifluoroacetic acid (TFA; solvent A) and acetonitrile:water (8:2) containing 0.1% TFA (solvent B). Elution was by step gradient from 90:10 (A:B) to 80:20 in 15 min, then from 80:20 to 60:40 in 10 min, and subsequently from 60:40 to 40:60 in 10 min, from 40:60 to 30:70 in 10 min. Flow rate 0.3 mL/min.

It is interesting to note that the fresh or lyophilised nature of the starting material does not influence the fingerprints of flavonoids and anthocyanins, allowing to suppose that it is possible to store the berries by lyophilisation for a delayed use for the preparation of the extract, in order to solve the problem of the seasonal nature of the berries that limit the production of the liqueur.

The ultrasonic extraction was investigated too but this extraction in our experimental conditions did not give any advantage, generating an extract with different chemical composition if compared to that obtained by following the traditional recipe.

3.2. Stability test

Phenolic compounds have an important role for the organoleptic properties of the finished product, in fact the colour and the taste of the extracts and of the finished liqueur are strongly dependent by these chemical constituents. Aggregation products of anthocyanin degradation are tannins that are able to confer a bitter taste to the food containing them.

Considering the results above described for extracts produced by sonication enhanced maceration, the stability test was performed only on the extract obtained by following the traditional recipe both on lyophilized and fresh material. Data obtained for the two extracts were homogenous, thus only the data relative to the extract obtained from fresh material are reported here.

In order to perform a stability test, a sample of 100 mL of the extract was transferred in a dark bottle, and stored in the dark for 1 year. Monthly, a sample of 1 mL was taken, dried under nitrogen, frozen and then stored. All the samples were analysed by reconstructing a solution in 1 mL of ethanol:water mixture, before the analysis.

In order to perform a quantitative analysis two internal standards were selected, one for building a calibration curve for anthocyanins and one for building a calibration curve for flavonoids. Cyanidin-3-*O*-galactopyranoside for anthocyans and rutine (quercetin-3-*O*-rutinoside) for flavonoids were selected as suitable internal standards, their retention times were not interfering with any of the compounds under investigation, and their UV response was similar to those of the corresponding external standards.

Calibration curves were built for all the 15 compounds, some of them available as commercial standards, other isolated in our laboratory in a previous experimental work. Validation of the method was realised in agreement with EMEA note guidance on validation of analytical methods [26].

Fig. 4 shows the evolution of anthocyanins (panel A), and flavonoids (panel B) in extracts of *M. communis* during an year of storage in dark of the extract prepared by following the recipe for the preparation of the liqueur.

Anthocyans are very poorly stable in the storage condition, most of the compounds result quantitatively lowered at the end of the year of storage, the quantitative value for all of them get down under the detection limit of the method.

After 6 months the quantity are halved and the initial concentration does not undergo a big change in quantity only during the initial 3 storage months.

Flavonoids are more stable, however their concentration lower in the time. Compound **10**, myricetin-3-*O*-rhamnoside, for example, that is the most abundant flavonoid in the extract reduces its concentration from an initial value of 1.7 mg/mL to a final value of 0.5 mg/mL at the end of the storage period. After the stability test period most of the flavonoidic components reduce their concentration to a halved quantity respect to the initial quantity. Some compounds lower their concentration more.

The above data permit to suppose a very scarcely stability of the phenolic fraction in hydroalcoholic myrtle extract, and to suggest to use the extract for preparing myrtle liqueur not over the 3 months after its preparation.

A better conservation system could be the lyophilisation of the berries that, as previously shown, allows the production of an extract with the same chemical composition of the extract obtained from the fresh berries.

3.3. Antioxidant activity

Previous studies have shown that flavonoids, have antioxidant or antiradical activities [3,4,24], which contribute to the explanation of the protective effect of vegetable-rich diets against coronary diseases. The antioxidant activity of plant extracts is correlated with their phenolic content [25]. Anthocyanins and flavonoids are major components in the myrtle berries and its extracts. TEAC assays indicated that the black *M. communis* berries had high radical scavenging activities, which can be attributed to an high level of anthocyanins and flavonoids.

Fig. 5 shows the results of the evaluation of the antioxidant activity, measured by TEAC assay, on the hydroalcoholic extract prepared by following the traditional recipe and its variability in time. Antioxidant activity is expressed as TEAC that is the Trolox concentration necessary for obtaining the same radical $ABTS^{+}$ inhibition of a solution 1 mg/mL of the extract under investigation.

The extract prepared from fresh berries (after 40 days of maceration) showed an interesting antioxidant activity, with a TEAC value of 0.915 ± 0.050 mg/mL. The value increased after 2, 3 months. This finding can be explained by hypothesising

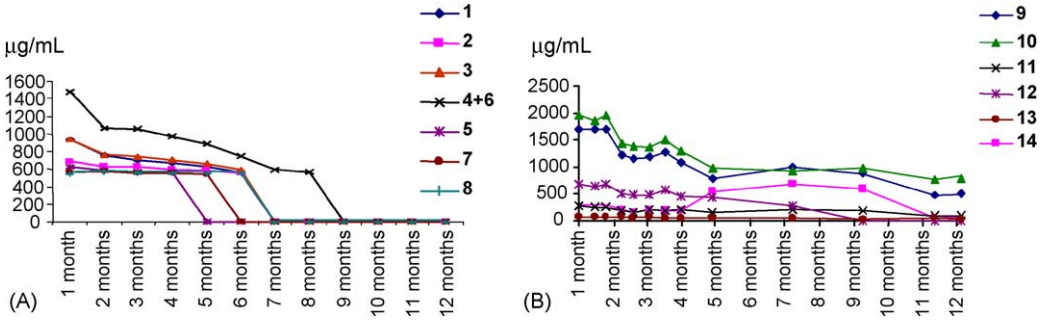


Fig. 4. An year storage stability tests for anthocyans (A) and flavonoids (B) in *M. communis* berry extracts.

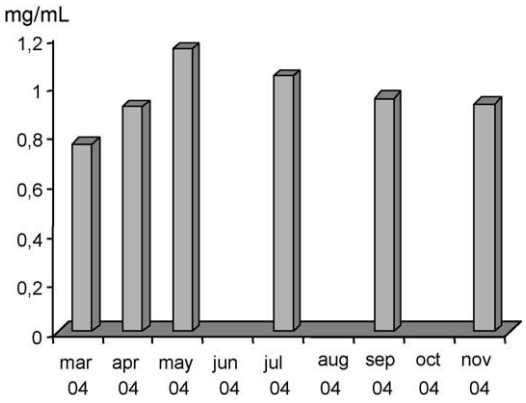


Fig. 5. Free-radical scavenging activity of myrtle extracts measured by TEAC assay, and relative variation during nine months of storage.

Table 1
TEAC values for myrtle hydroalcholic extract, among the time of stability test

Months	TEAC	DS
1	0.915	0.050
2	0.964	0.081
3	1.154	0.072
5	1.042	0.110
7	0.952	0.102
9	0.930	0.091

an hydrolysis of the flavon glycosides, that gives result as an additional hydroxyl group able to participate to the redox reaction with the cation radical. By observing the stability curves of flavonoids it is possible to note a decrease of flavonoid glycoside contents in the same time. After this time the TEAC value is stable and starts to decrease in little measure, probably due to the degradation of anthocyans after a long storage time. After 8 months the activity can considered stable at a value of TEAC very similar to that of the fresh extract (0.930 ± 0.091 mg/mL).

In Table 1 and in Fig. 5 the TEAC values of a myrtle extract measured during 9 months are shown.

4. Conclusions

Phenolic compounds, flavonoids and anthocyans are the major phytochemicals in *M. communis* berries, and in the extracts prepared following the traditional Italian recipe for the preparation of myrtle liqueur.

Berries have a seasonal nature, thus it is important to study conservation strategy for berries or their extracts. In this report the evolution of flavonoids and anthocyanins in myrtle extract was investigated showing that extracts are very unstable and their phenolic composition is preserved only in the initial 3 months of the storage period.

On the other hand lyophilisation appears as a considerable way for the storage of the berries.

Antioxidant activity, evaluated as free-radical scavenging ability, presents high value and is stable in time. The high antioxidant activity is in agreement with the high content of phenolic compounds in berries.

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