Journal homepage: www.fia.usv.ro/fiajournal
Journal of Faculty of Food Engineering,
Ştefan cel Mare University of Suceava, Romania
Volume XV, Issue 2 - 2016, pag. 123 - 130

EVALUATION OF ASCORBIC ACID AND PHENOLIC CONTENT OF FOUR TRADITIONAL ROMANIAN MEDICINAL BERRY SPECIES

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Received 29thMarch2016, accepted 17thMay 2016

Abstract: *This paper analyzes the total phenolic compounds and ascorbic acid in water, methanol extracts from four selected wild fruits originally from the Bukovina area, namely, sea-buckthorn (Hippophae rhamnoides L), cranberry bush (Viburnum opulus L.), hawthorn (Crataegus monogyna L.), and rosehip (Rosa Canina L.), used as tea beverages or as decoction for medicinal purposes.*
Ascorbic acid was separated, identified and dosed in fruit extracts by means of high-performance liquid chromatography (HPLC); the total phenolics were determined as gallic acid equivalents GAE/100 g FW, by the Folin-Ciocalteu method. The levels of berries mean ascorbic content in the four different families ranged from 112.87 (Viburnum opulus L) to 224.21 mg/kg of fresh weight (Crataegus monogyna L.). The total phenolic content ranges from 4.38 mg GAE/kg FW (Hippophae rhamnoides L) to 11.09 mg GAE/kg FW (Crataegus monogyna L.).
These results suggest that the Crataegus monogyna L. extract is a rich source of natural antioxidants.

Keywords: *drying, freezing, sea-buckthorn, cranberry bush, hawthorn, rosehip, ascorbic acid.*

1. Introduction

The consumption of forest fruits has beneficial therapeutic effects such as hypotensive, anxiolytic and sedative and it reduces the risk of contaminating cardiovascular diseases, some cancers, lung diseases. Furthermore it has anti-oxidant and immune enhancing effects. These properties are attributed to a variety of constituents, including micro and macronutrients such as flavonoids, tannins, sugars and organic acid (citric and malic acid, ascorbic acid and phenolic acids), vitamins and minerals.
Sea buckthorn grows in the form of a shrub. Buckthorn plants are very resistant to frost and drought. The fruits are orange and have an astringent, sour, but pleasant taste.
In Romania sea buckthorn berry is widespread. It is harvested from spontaneous flora and has been used for medicinal and nutritional purposes. Sea buckthorn's natural distribution area includes China, Mongolia, India, Nepal, Pakistan, Russia, Latvia, Romania, Great Britain, France, Denmark, Netherlands, Germany, Poland, Finland, Sweden, Norway and Canada. *Hippophae rhamnoides*has been further divided into eight subspecies [1]. The fruits with a rich oil content in seeds, contain fat soluble vitamins (A, K, and E), fatty acids, lipids, organic acids, amino acids, carbohydrates, vitamins C, B_1, B_2, folic acid, tocopherols and flavonoids, phenols, terpenes and tannins [2]. Sea buckthorn berry is an excellent source of vitamin C, approximately 400 mg/100 g [3], fat soluble vitamins, antioxidants [4] and bioactive phenolic compounds that both individually and synergistically may help protect against cardiovascular diseases,

cancer, inflammation, obesity, diabetes, skin diseases, and other chronic diseases. Fructose and glucose were the major sugars in sea buckthorn berry, and the dominating acids were malic and quinin acids [5]. The total anthocyanin content of sea buckthorn was negligible (0.84 mg/100 g) [6].

The orange color of fruits of sea buckthorn is also due to the large content of carotenes that are powerful antioxidants. Sea buckthorn are used to prepare juices, oil, jellies, marmalade, fruit pulp, ice-cream, vitamin C tablets, liqueur, wine, alcoholic beverages, tincture or decoction. Cosmetic applications for sea buckthorn include moisturizing, dandruff control and hair loss prevention lotions, anti-ageing skin creams and lotions, and sun care cosmetics [7].The fruits used for medicinal purpose are collected after cooking and can be used either fresh or dried. Their drying is done naturally or artificially (50 – 60°C). Fruits may be kept frozen, a process carried out in two stages (at 1-2 °C, then at - 35-36°C).

The cranberry bush (*Viburnum opulus L.)* grows as a bushy shrub, and in Romania is found in moist woods and thickets, from lowland and hill, but also in the alpine and sub alpine areas. In autumn, the plant makes red fruit and the leaves change their color in red. The flowers are pale green with a diameter of 5-7 cm bud blooms in May-June. The fruits are red, fleshy and spherical with a diameter of 8 mm and contain one flat seed, grow in September and have a sour-bitter like flavour. The flowers, fruits and the crust of the cranberry bush contain valeric acid and tannin and is used in the pharmaceutical industry and the preparation of antispasmodic drugs and other drugs.

The fruits contain: ascorbic acid, citric acid, B, C, K, P, vitamins, esters, oxalic acid, free and esterified sterols that are important to human nutrition [8]. The fruits are sometimes used as syrups, tinctures, decoct jellies and marmalade.

Rosehip (*Rosa Canina L.*) is a 1-3 m shrub that has spikes, a wide base and curved tip down sickle. The flowers are pink, sometimes white 2-3, located at the tip of the branches.

The fruit – the globular or ovoid receptacle is red-orange or even deep red depending on the stage of ripeness [9].

Rose hip fruits are rich in carotenoids which vary depending on harvesting time [10], and are considered a particularly rich source of ascorbic acid [11, 12]. The fruits contain biologically active components such as vitamin A, B complex, E, also minerals like Mg, K, S, Ca, Fe, Se, Mn and Si [13], polyphenols [14], amino acids, citric acid, malic acid, flavonoids, pectin, lecithin, sugars, tannins, and volatile fatty oil. Carotenoids provide immunological and antimutagenic protection and prevent tumors [15]. Vitamin C supplementation is known to have a protective effect against several disease conditions, most notably the common cold. It can strengthen the immune system against some infections, cardiovascular diseases and some cancers [13, 16].

Hawthorn *(Crataegus monogyna Jacq)* has special qualities in treating heart disease and is known from ancient times for its therapeutic effects. It grows like bushes, especially at the border of mountain forests, low land and pastures, sometimes grown for ornamental purpose [17]. Hawthorn fruit can be eaten fresh (which are available in this form) but the decoction of dried fruits can be made jam, marmalade and liquors, mixed with other herbs or added to some chicken or fish dishes.

The hawthorn fruits contain vitamin C, flavonoids, tannins, tartaric acid, ursolic, citric, oxalic, nicotinic acid, chlorogenic acid, choline acetyl choline, pectin, glucose, fructose, sucrose, maltose and minerals [18, 19]. Hawthorn fruits are used

to produce jams, jellies, juices, alcoholic beverages and other drinks [21].
The objective of the present study was to investigate the antioxidant activity and study the possible interdependence of a series of wild berries fruits commonly used in the folk medicine in Bucovina (Romania). The fruits are preserved fresh, dried and frozen and are used as teas or decoctions, for the treatment of several diseases (Table 1).

2. Materials and methods

2.1. Solvents and reagents

Reagents and solvents were procured from Sigma Chemical. Deionizer water was used throughout.

2.2. Plants source and extracts preparation

The berry fruits were collected at the fully ripe mature stage (late September and October) 2014 from hillsides and hedgerows in Cacica Forest, located in western part of the Bucovina Region in Romania.

Table 1

The studied botanical species and their traditional uses

Plant name	***Common name***	***Traditional uses***
Hippophae rhamnoides L	sea-buckthorn	Cardio tonic, anti-inflammatory
Viburnum opulus L	cranberry bush	asthma, antitussive, bronchial diseases and coughs
Crataegus monogyna L.	hawthorn	Sedative, hypotensive, antiarhytmic, heart palpitation
Rosa Canina L.	rosehip	Cardiotonic,vitaminic, antioxidant, antiinflammatory

The fruits were sorted visually for color (maturity), size and physical damage in the laboratory. The test samples were divided into three equal parts as follows: the first one was packed into plastic bags and was stored in a refrigerator at 4^0C overnight; the second part of fruits was dried in the natural form and the third one was kept in a freezer at −20°C.

2.3. Chemical analyses

The initial moisture content of fruits was determined according to the European Standard EN ISO 665/2000 by drying at the temperature of 103 °C.
Total ash composition was obtained by calcinations of 5g of sample at 600 °C for 240 min.
Protein content was analysed by using the Kjeldahl method.
Total phenolics were measured in duplicate samples of each extract, using the Folin-Ciocalteu method: Briefly 1.0 mL of the extracted sample, 10 mL of Folin−Ciocalteu reagent, and 9 mL 7.5% $Na_2 CO_3$ solution was added. The solutions were mixed in the cuvette by using a pipet and allowed to equilibrate at room temperature for 2 hours. The absorbance was recorded at 750 nm using a spectrophotometer T70 UV-VIS PG Instruments Ltd. The results are expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh weight (FW) of fruit material.
Ascorbic acid determination was done on acid extracts of samples.
Extraction of ascorbic acid from samples
The extracts were obtained following the protocol: 4 gram of fruit was extracted with 12 ml of acidified solutions (Perchloric acid and o- Phosphoric acid 1%) using a ceramic mortar and a pestle. The residue was re-extracted until the extraction solvents remained colorless (the total solvent volume was 50 ml).
The extract was filtered through a Whatman no. 5 filter paper. The extracts were kept at -20°C until further analysis.
Ascorbic acid separation, identification and dosage

The ascorbic acid in the samples was separated, identified and dosed in a HPLC SHMADZU system coupled with UV–VIS detector (DAD). ZORBAX -C18 column (5µm, 250x4.6) was used. The column was eluted in isocratic system with a mobile phase that consisted of phosphate buffer pH = 3.5 (TFA): solution 0.02 m/l of monopotassium phosphate and orthophosphoric acid 10%, adjusted to pH = 3.5. The pump flow rate was set at 0.6 ml/min. The chromatograms were registered at 245 nm.
For ascorbic acid identification standard L-ascorbic acid (Sigma 99% standard L ascorbic acid) was used. For dosage of ascorbic acid in the samples, a calibration curve was constructed using dilutions of standard L-ascorbic acid in bidistilled water [20].

2.4. Statistical Analysis

Statistical analysis was done using the *Analysis* ToolPak of M.S. Excel 2010. Two factor ANOVA without replication was used for analysing the significant differences of ascorbic acid concentrations and total phenolics mg GAE/100 g of fresh fruits, respectively air dried and frozen wild berries fruit samples. All samples were drawn from normally distributed populations having a common variance. For this statistical analysis the samples were drawn independently from each other.

3. Results and Discussions

Table 1 shows the values obtained for crude protein, ash, moisture, ascorbic acid and total phenolics content of fruit samples.

Macronutrients

The data presented as a preliminary study on protein content of sea-buckthorn, cranberry bush rose hip and hawthorn from the Romanian ethnoflora, shows these fruits were not a very rich source of proteins showing maximum values of 7.68 g/100g fw (*Rosa Canina L.*) and 6.36 g/100 g fw (*Crataegus monogyna Jacq*) (Table 2).
The proteins contained in fruits contribute to the regeneration of tissues.
The *Viburnum opulus L.* and *Hippophae rhamnoides L.* fruits reach the highest values of moisture (75.66 respectively 72.34 %) compared to those of *Rosa Canina L.* and *Crataegus monogyna Jacq.* Alternatively, the *Hippophae rhamnoides L.* fruits attain the lowest values of ash 1.099%. The significance of the drying condition is mentioned in Table 2.

Table 2

Moisture (g/100 g of fresh weight), protein content (g/100 g of dry weight) and ash content of fruits used for analysis

	Samples	***Moisture%***	***Protein content % S.U.***	***Ash%***
sea-buckthorn	*Hippophae rhamnoides L.*	72.34	4.3	1.099
cranberry bush	*Viburnum opulus L.*	75.66	4.87	1.3997
rosehip	*Rosa Canina L.*	46.76	7.68	1.8426
hawthorn	*Crataegus monogyna Jacq*	55.49	5.36	2.5737

Unal, H. Guran, and KamilSacilik [18] found that in the case of hawthorn fruits dried at 50, 60 and 70^0 C air temperatures, the air temperature and blanching in hot

water had a significant effect on the moisture content of samples.
In contrast to animal protein that favours the atherosclerosis and the accumulation of cholesterol in the blood plasma; vegetable protein contribute to maintaining low cholesterol levels.
The impact of frozen storage and air drying on ascorbic acid content was evaluated and compared to the ascorbic acid content in fresh samples that were frozen and stored at -20^0 C for six months.

Ascorbic acid C level in fresh berry varies between 112.87 (cranberry bush) and 224.21 mg/100 kg FW of fruits (hawthorn), whereas for rosehip the concentration is 216.62 mg/kg of fruit and for sea-buckthorn, 222.25 mg/kg of fruit (Figure 1). **The total phenolics content** determined by spectrophotometric methods for the mentioned fruits is depicted in the Figure 2.

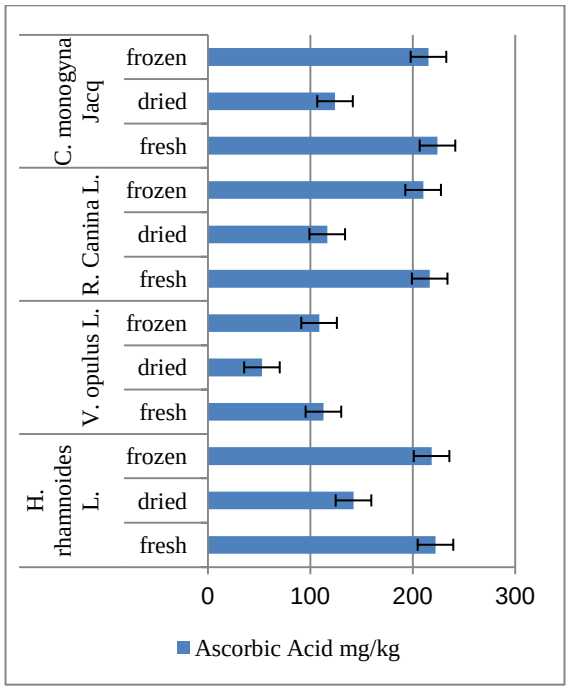

Figure1. The ascorbic acid concentrations in fresh, Air dried and frozen wild berries fruit samples

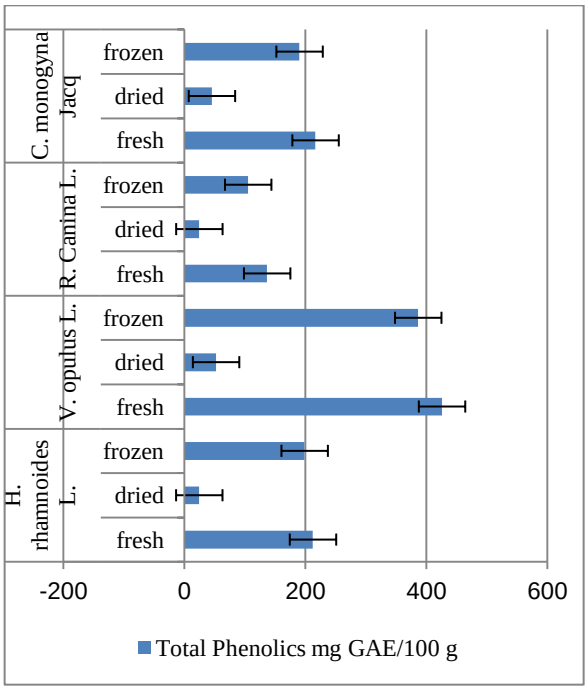

Figure2.Total phenolics variation content in fresh, air dried and frozen wild berries fruit samples

Frozen samples presented a slightly higher reduction in ascorbic acid content than fresh fruits. However, the difference was not statistically significant. Vitamin C is a dietary nonenzymatic antioxidant required as a co-factor for many enzymes and can be obtained exogenously, as a part of a diet rich in wild berries. Other reports show rosehip fruits pulp contains between 53 and 563 mg/100g fresh substances [21]. Comparatively, Leahu A et al., [20] found the rosehip jam is rich in vitamin C; the products analyzed ranging from 37 mg/100 g and 32.25 mg/100 g [20].
These results are in agreement with other literature data, such as: Roman et al., [22] who obtained 360.22 mg/100 g frozen pulp (var. *transitoria f. ramosissima*, altitude 1250 m), Ropciuc et al., [23] who obtained values ranging from 374.12 mg/100g and 663 mg/100g rosehip powder, and Barros, Lillian, et al., [24], who reported 220.24 mg/100 g dry weight in *Crataegus mono gyna* ripened fruits. Sea-buckthorn and cranberries have a great importance in

strengthening human immunity; this effect have been associated with the presence of vitamin C.

The poliphenols' content varies, only the cranberry bush has higher content (Figure 3). The content of polyphenols in buckthorn, hawthorn and rosehip is correlated with the content with vitamin C.

All these fruits have a prophylactic and therapeutic activity against a wide range of diseases, including the inflammatory, flu, colds and infection. Therefore, the overall drying process, had a negative effect on the phenolic contents of sea buckthorn, reducing with 88.51%, similar result were obtain for cranberry bush, total phenolics content is decreasing with 87.75%. On the other hand, drying process had a greater effect on total phenolics loss in rosehip (82.04%) and hawthorn (79.09%). Total phenolic contents of frozen samples were found stable over 1 month of storage (figure 3). The fruits, which were stored frozen for 1 month, showed no significant difference in total phenolic content from the fresh berries.

Lillian Barros et al., [24], also reported the phenolic were the major antioxidant components (247.03-701.65 mg GAE/g 393 of extract); unripe fruits revealed the highest content in phenolics. Rosu C. M. et al. [11] founded that the ascorbic acid content in rose hips decreased after four month freezing, compared with the level quantified in the fresh fruits.

Using two factor ANOVA without replication we analyzed if there exist significant differences for ascorbic acid concentrations and respectively, total phenolics (mg GAE/100 g) for fruits in fresh, air dried and frozen wild berries fruit samples. There are two null hypotheses: one for the rows which contain the Factor A (fruit samples fresh, air dried and frozen wild berries) and the other for the columns which contain the Factor B (type of fruits used for analysis: sea-buckthorn, cranberry bush, rosehip and hawthorn, respectively. We note by H_0 the null hypotheses which consist in the following assumption: there is no significant difference in yield between the (population) means of the blends.

The results regarding significant differences for ascorbic acid concentrations and respectively, total phenolics (mg GAE/100 g) are given in Table 3.

Table 3

Analysis of variance for ascorbic acid and phenolic content in wild berries (factor B), used fresh, air dried and frozen (factor A)

		Total Ascorbic acid		**Total phenolics mg GAE/100 g**	
Source	**d.f.**	**MMS**	**F-value**	**MMS**	**F-value**
Factor A	2	9021.098	80.17***	52667.23	12.90**
Factor B	3	7048.312	62.64***	21497.9	5.26*
Error	6	112.5194	-	4081.219	-

d.f. = Degree of freedom; MSS = Mean sum square; Significant at 5% level (p*), 1% (p**) and 0.1% (p***) .

Since the p-value (Factor A - samples fresh, air dried and frozen wild berries) = $4.69 \oplus 10^{-5} < 0.001 = \alpha$, we can reject the Factor A null hypothesis and we conclude (with 99.9% confidence) that there are significant differences between ascorbic acid concentrations in fresh, air dried and frozen wild berries fruit samples (table 3). Also, F = 80.17 > 5.14 = F-critic we reject the null hypothesis, and conclude that ascorbic acid concentrations for fruit samples - fresh, air dried and frozen wild

berries are statistically different. Since the p-value (Factor B -type of fruits used for analysis) = 6.4 $\oplus 10^{-5} < 0.001 = \alpha$, we can reject the Factor B null hypothesis and we conclude (with 99.9% confidence) that there are significant differences between ascorbic acid concentrations for fruits used for analysis (Table 3).

Also, $F = 62.64 > 4.75 = F\text{-}critic$ we reject the null hypothesis and conclude that ascorbic acid concentrations for types of fruits used for analysis are statistically different.

Since the p-value (Factor A - samples fresh, air dried and frozen wild berries) = $0.0067 < 0.05 = \alpha$, we can reject the Factor A null hypothesis and we conclude (with 95% confidence) that there are significant differences between total phenolics mg GAE/100 g for fruits used for analysis in fresh, air dried and frozen wild berries fruit samples (table 4). Also, $F = 12.90 > 5.14 = F\text{-}critic$ we reject the null hypothesis, and conclude that total phenolics mg GAE/100 g for fruits samples - fresh, air dried and frozen wild berries are statistically different. Since the p-value (Factor B -type of fruits used for analysis) = $0.04 < 0.05 = \alpha$, we can reject the Factor B null hypothesis and we conclude (with 95% confidence) that there are significant differences between total phenolics mg GAE/100 g for fruits used for analysis (table). Also, $F = 5.26 > 4.75 = F\text{-}critic$ we reject the null hypothesis and conclude that total phenolic mg GAE/100 for fruits used for analysis are statistically different.

4. Conclusions

All analyzed fruit samples in this study represent a significant source of phenolic compounds and ascorbic components that together bring a huge benefit to the human body, fight free radicals, chronic diseases and are revitalizing strong. The results of this study indicate that the contents of ascorbic acid in hawthorn fresh fruit were higher than in cranberry bush.

The ascorbic acid concentrations and the total phenolics mg GAE/100 g, respectively for fruit samples (fresh, air dried and frozen wild berries) and, respectively for types of fruits (sea-buckthorn, cranberry bush, rosehip, hawthorn) are statistically different and we remark that slightly higher reduction in ascorbic acid content was observed in frozen samples than in fresh fruits, but the overall drying process had a negative effect on the phenolic contents of these fruits. The cranberry bush presents higher content of polyphenols, whereas for the other studied fruits it varies. For buckthorn, hawthorn, rosehip, the content of polyphenols is proportional with the content of vitamin C.

The present study revealed the high content of total phenolic content in cranberry bush (*V. opulus L.)* from Bucovina Region in Romania and recommend them as excellent source of antioxidants.

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