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The effect of freezing on the antioxidant activity of Bulgarian *Chrysanthemum balsamita*

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ABSTRACT

The aim of the present paper was to investigate the antioxidant capacity and the possible cell structure changes of frozen *Chrysanthemum balsamita* leaves. The antioxidant activity of the studied aqueous extracts was estimated through ABTS, DPPH, FRAP, and CUPRAC methods. The total phenolic content was assessed as well. The results varied significantly before and after freezing of the plant samples. The total phenolic content of the fresh leaves was established to be 0.59 mg GAE/g PW and 0.68 mg GAE/g PW of the frozen leaves.
The microscopic analysis of both fresh and frozen leaves showed changes in the cellular structure which influenced the radical scavenging activity of the frozen *Chrysanthemum balsamita* plant samples.

Key words: *Chrysanthemum balsamita*, costmary, antioxidant activity, cell structure

Introduction

Aromatic, medicinal and spicy plants are an immense and sustainable source of natural compounds with various beneficial properties. They have been used since ancient ages for various applications, particularly healing of diseases, flavoring of foods and formulation of fragrances. Some of these plants nowadays are grown commercially and are used for the production of a variety of ingredients.

Costmary (*Chrysanthemum balsamita* L.) is a medicinal plant, belonging to the Asteraceae family. It is a large perennial plant of Asian origin with yellow flowers, grown in Europe and Asia since the Middle Ages (Bylaitė et al., 2000). This plant has a hairy stem, complete shiny leaves, highly branched from the base and 70-120 cm height (Nickavar et al., 2003; Mozaffarian, 2004; Hassanpouraghdam et al., 2009). It has a characteristic odor due to its volatile oil constituents. The main compounds (above 3%) are: carvone (47.81%), α-thujone (12.56%), germacrene B (5.23%), benzaldehyde (4.64%) ethylbenzene (3.96%) and germacrene D (3.13%). Costmary has different phytochemicals such as; oxygenated terpenes (73.5%), hydrocarbon terpenes (14.6%), aromatic hydrocarbons (5.7%), aromatic oxygenated (4.7%) and other components (1.5%) (Sapundzhieva et al., 2013). It is also rich in phenylpropane derivatives, flavones, sesquiterpene lactones, tannins and oligoelements (Gallori et al., 2001). Costmary has been used as a spice for flavoring different types of food, cakes, drinks, as well as in confectionery since antiquity (Bylaitė et al., 2000; Gallori et al., 2001). A herbal tonic tea of Costmary leaves traditionally is consumed in various cultures (Abad et al., 2006). Information on the effect of some technological factors on the antioxidant activity of plant extracts is scares.

The objective of this study was to analyze the extracts of fresh and frozen *C. balsamita* leaves for their radical scavenging activity and the effect of the expected cellular structure changes of the herb on its phenolic content and antioxidant activity.

Materials and Methods

Plant material

Chrysanthemum balsamita L. plant samples were collected in May 2013 from their natural habitat in Plovdiv region, Bulgaria.

Extract preparation

The applied method was decoction – extracting by boiling fresh/frozen/dried herbal and plant material. 2.5 g of the fresh and frozen plant sample were boiled for 15 min in 50 ml of distilled water. The resulting solution was being filtered before analyzed.

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Determination of total polyhenolic content (TPhC)

A modified method (Kujala et al., 2000) was used for the determination of the total polyphenolic content (TPhC). Each extract was mixed with Folin-Ciocalteu reagent and 7.5% Na_2CO_3. The mixture was vortexed and left for 5 min at 50°C. After incubation, the absorbance was measured at 765 nm and room temperature. Gallic acid was employed as a calibration standard and the results were expressed as mg gallic acid equivalents (mg GAE) per gram of plant weight.

Determination of antioxidant activity

DPPH$^{\bullet}$ radical scavenging assay

Antioxidant activity was described as having activity against the stable form of the synthetic product DPPH$^{\bullet}$ (2.2-diphenyl-1-pikrylhydrazil) by the method of Brand-Williams et al. (1995) with slight modifications. A freshly prepared 14.10^{-5} M solution of DPPH$^{\bullet}$ (in methanol) was mixed with the sample in a ratio of 2:0.5. The unit of Trolox equivalent antioxidant capacity (TEAC) defined the concentration of Trolox having equivalent AOA expressed as μM TE/g PW.

ABTS$^{\bullet+}$ radical scavenging assay

The radicals scavenging activity of the ultrasound extract against radical cation (ABTS$^{\bullet+}$) was estimated according to a previously reported procedure with some modifications (Re et al., 1999). ABTS$^{\bullet+}$ was produced by reacting 7 mM of ABTS$^{\bullet+}$ solution with 2.45 mM of potassium persulphate, and the mixture was kept in the dark at room temperature for 12-16 h. At the moment of use, the ABTS$^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30°C. One ml of ABTS$^{\bullet+}$ solution was added to each sample (0.01 ml) was vigorously mixed. After reacting at 30°C temperature for 6 min, the absorbance at 734 nm was measured. The TEAC value was defined as the concentration of Trolox having equivalent antioxidant activity expressed as μM TE per gram plant weight (μM TE/g PW).

Ferric-reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the procedure of Benzie and Strain (1996) with slight modification. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe (II)-tripyridyltriazine compound from colorless oxidized Fe (III) form by the action of electron donating antioxidants. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37°C in a water bath prior to use. One hundred and fifty microliters of plant extracts were allowed to react with 2850 μl of the FRAP reagent solution for 4 min at 37°C and the absorbance of the reaction mixture was recorded at 593 nm. The results were expressed as μM TE/g PW.

CUPRAC assay

The CUPRAC assay was carried out according to the procedure of Apak and coworkers (Apak et al., 2004) with modifications. To a test tube were added 1 ml of $CuCl_2$ solution (1.0×10^{-2} M), 1 ml of neocuproine methanolic solution (7.5×10^{-3} M), and 1 ml NH_4Ac buffer solution (pH 7.0), and mixed; 0.1 ml of herbal extract (sample) followed by 1 ml of water were added (total volume = 4.1 ml), and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of herbal extracts was measured as μM TE/g PW.

Microscopic analysis

Thin sections of the plant leaf were prepared by hand-cutting at a 40-45° angle of the cutting knife. The section should be as thin as possible. It was mounted on a microscope slide using tweezers. The thinner the cut the less the section curled (liquid may be applied for retrieving a better sample). A cover glass was pressed carefully either by hand or with tweezers (no air should be left between the slide and the cover glass). Photomicrographs of x400 magnifications were taken with Celestron Deluxe LCD Digital microscope and analyzed.

Statistical analysis

All measurements were carried out in triplicates. The results were expressed as mean ± SD and statistically analyzed using MS-Excel software.

Results and Discussion

Total phenolics

The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent was expressed in terms of gallic acid equivalents (Table 1). Freezing seams to affect *TPhC* of the examined extracts showing values of the fresh leaves 0.59 mg GAE/g PW and of the frozen samples - 0.68 mg GAE/g PW.

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Table 1. *Total phenol content (mg GAE/g PW) and in vitro antioxidant activity (μM TE/g PW) of Chrysanthemum balsamita aqueous extracts.*

Methods/ Plant sample decoction	TPC	$TEAC_{ABTS}$	$TEAC_{DPPH}$	$TEAC_{fRAP}$	$TEAC_{CUPRAC}$
Fresh leaves	0.59 ± 0.04	10.68 ± 0.25	3.55 ± 0.01	7.35 ± 0.12	5.93 ± 0.07
Frozen leaves	0.68 ± 0.01	12.01 ± 0.07	3.02 ± 0.01	8.79 ± 0.28	6.22 ± 0.09

Antioxidant activity

The antioxidant activity of aqueous extracts of *Chrysanthemum balsamita* was assessed with the use of four different assays: $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, FRAP and CUPRAC. These assays are widely used for determination of total AOA in many foods such as fruits, vegetables and spices (Prior et al., 2005; Ciz et al., 2010).

In order to investigate the antioxidant activity of the plant extracts, experiments with two stable radicals $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were conducted. A higher TEAC value indicates stronger antioxidant activity. The results represented in Table 1 revealed that all extracts possessed free radical-scavenging activity but at different levels.

Significant ABTS free radical scavenging activity was evident in the 15 min decoction of frozen leaves (12.01 ± 0.07 μM TE/g PW). The antioxidant potential of *C. balsamita* extracts was estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II) as well. In accordance with the ABTS assay and the total polyphenolics, the highest value in the FRAP method was obtained in the decoction extract of frozen costmary leaves - 8.79 ± 0.28 μM TE/g PW. The cupric ion (Cu^{2+}) reducing ability of the extracts of *C. balsamita* leaves was also evaluated. Among the two extracts, the decoction of frozen leaves showed the higher CUPRAC value – 6.22 ± 0.09 μM TE/g PW. The results of this assay correspond well to the already mentioned results pursuant to the ABTS, FRAP and TPC methods. Contrary to all the other conducted studies the DPPH assay confirmed the higher values established by the decoction of fresh leaves (3.55 ± 0,01 μM TE/g PW).). In comparison, the $TEAC_{DPPH}$ value for the frozen leaves was 3.02 ± 0.01 μM TE/g PW. This is probably due to the different mechanism of contribution of each individual component to the total radical scavenging activity of the studied samples.

Microscopic analysis

The surface of the fresh leaves was characterized by a mosaic structure (Figure 1). Under the microscope the outer edges had irregular shape and were transparent. Cell walls were distinguished in the fresh leaves (Figure 2). Fine transparent hairs can be seen in the middle green part of the fresh and frozen leaves (Figure 3, Figure 4). In the examined material of the frozen leaves structure changes were visible (Figure 5). Changes were spotted in the stems as well (Figure 6, Figure. 7). Further analysis would have to clear the mechanism and how freezing (-18°C) structural changes influence the radical scavenging ability of *C. balsamita* leaves. Probably changes occurred at the cellular level which altered the structure of the cells and the extraction of chemical substances. It was assumed that by freezing water forms crystals that mechanically damaged cell walls. Protein denaturation and aggregation, inactivation of enzymes in chloroplasts and mitochondria and increased fluidity of membrane lipids is likely to affect membrane permeability. Probably ruptured cell walls have led to a more complete extraction of the cell consisted substances.

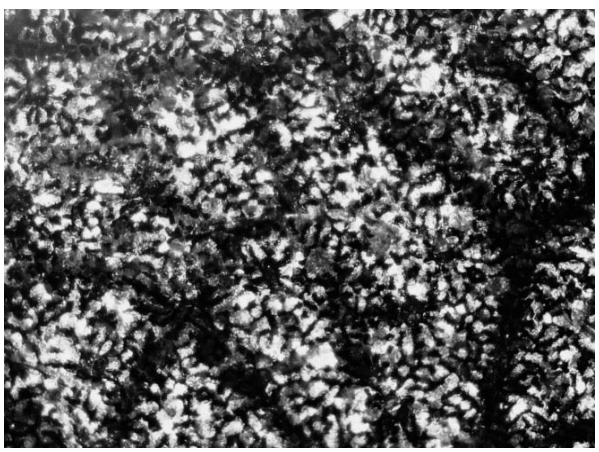

Figure 1. *Microscopic image of surface structure of fresh costmary leaf.*

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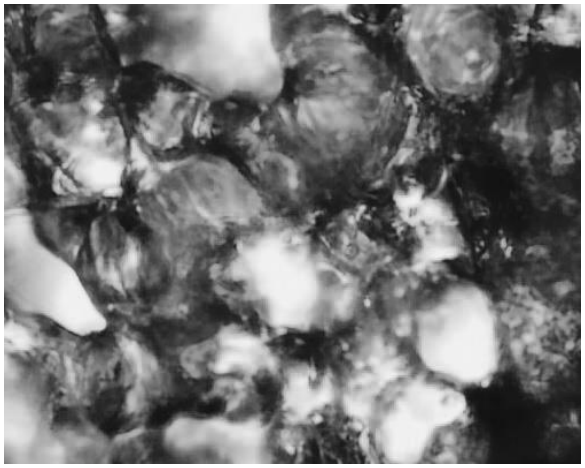

Figure 2. *Microscopic image of fresh costmary leaf.*

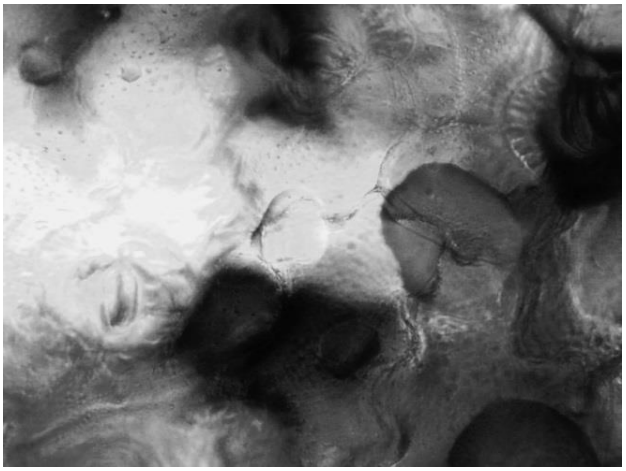

Figure 5. *Microscopic image of frozen costmary leaf.*

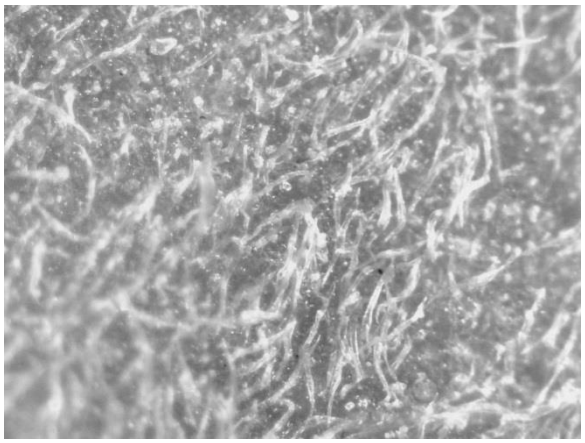

Figure 3. *Microscopic image of middle part of fresh costmary leaf.*

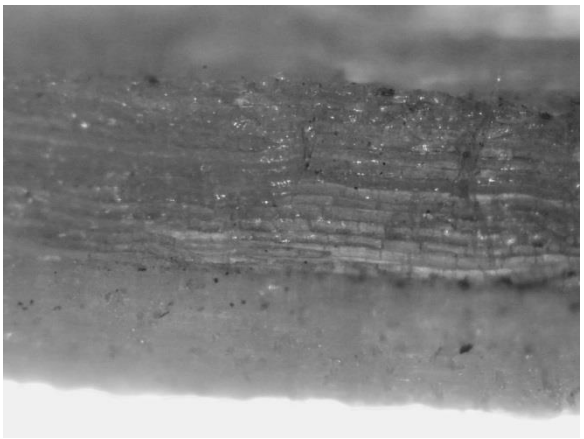

Figure 6. *Microscopic image of fresh costmary stem.*

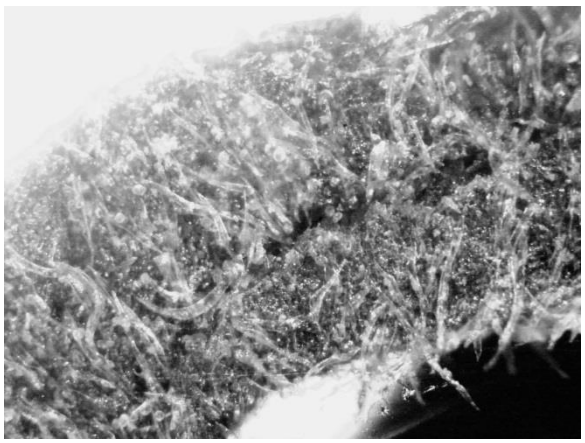

Figure 4. *Microscopic image of middle part of frozen costmary leaf.*

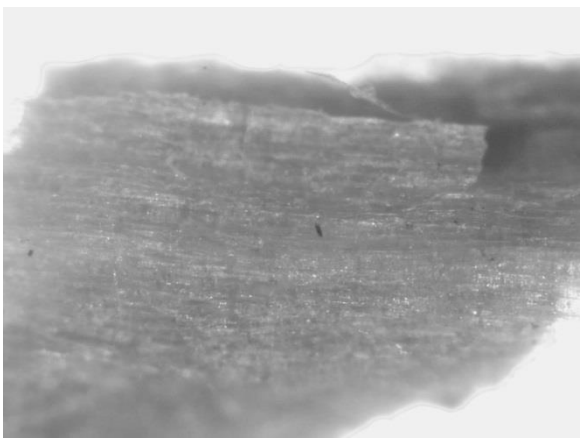

Figure 7. *Microscopic image of frozen costmary stem.*

Conclusion

Costmary shows a great potential as an ingredient of foods containing compounds with antioxidant activity. Freezing fresh plants is a common way of storing used in the food technology. Being stored in a freezer the costmary leaves are very likely to experience cell changes leading to a higher radical scavenging capacity.

Acknowledgment

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