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## Content of Phenolics, *in vitro* Antioxidant Activity and Cytoprotective Effects against Induced Haemolysis of Red Cabbage Extracts

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**SIMONA OANCEA<sup>1</sup>, LIDIA MILA<sup>2</sup>, OTTO KETNEY<sup>1</sup>**

<sup>1</sup>„Lucian Blaga” University of Sibiu, Department of Agricultural Sciences and Food Engineering, Sibiu, Romania

<sup>2</sup>„Lucian Blaga” University of Sibiu, Department of Environmental Sciences, 550012 Sibiu, Romania

\* *Corresponding author*: SIMONA OANCEA, „Lucian” Blaga University of Sibiu, Department of Agricultural Sciences and Food Engineering, 7-9 Ion Ratiu Street, 550012 Sibiu, Romania, Tel.: +40269-211338, Fax: +40269-212558, E-mail: [simona.oancea@ulbsibiu.ro](mailto:simona.oancea@ulbsibiu.ro)

### Abstract

Consumption of plant foods has been associated with a reduced risk of several diseases including cardiovascular and cancer. This is supported by their content of phytochemicals which provide a variety of pharmacological properties. The aim of the present study was to evaluate the content of antioxidant compounds with polyphenolic structure from seven different red cabbage samples, as well as their antioxidant and antihaemolytic activity.

The mean values of total anthocyanins and flavonoids of the investigated ethanolic and acidified ethanolic extracts were 1228.81 mg 100g<sup>-1</sup> DM and 2402.27 mg quercetin 100g<sup>-1</sup> DM, respectively. The results indicate a positive correlation of anthocyanins with flavonoids. We report strong antioxidant activity of the crude extracts as measured by relevant assays such as Folin-Ciocalteu and ferric reducing antioxidant power (FRAP). Good positive correlations were found between FRAP and flavonoids, and between phenolics and anthocyanins, respectively. We also describe the cytoprotective effects of the ethanolic crude extracts against free-radical induced oxidative damage in erythrocytes.

The present study demonstrates the high content and activity of antioxidant compounds in red cabbages from different geographic regions. In addition, it provides the first scientific evidence for a protective role against cytotoxic effects of reactive oxygen species in erythrocytes linked to different sample sources.

**Keywords:** red cabbage, anthocyanins, phenolics, antioxidant, induced haemolysis

## 1. Introduction

Oxidative stress, which is induced in human body by reactive oxygen species (ROS), plays a significant role in the development of several chronic diseases, *e.g.* cardiovascular, neurodegenerative, arthritis, autoimmune diseases, diabetes, and cancer. ROS, such as superoxide radical  $O_2^-$ , hydroxyl radical  $HO\cdot$ , hydrogen peroxide  $H_2O_2$ , and singlet oxygen  $^1O_2$  mainly produced in mitochondria, may lead to: lipid peroxidation in cell membranes, enzyme inactivation, activation of particular signalling pathways, DNA damages, generation of toxic compounds. Oxidative stress is produced as a result of an impaired balance between oxidative conditions and antioxidant mechanism. The rate of oxidative stress is related to the overall production of ROS, the efficiency of the endogenous antioxidant defence mechanism and the level of exogenous antioxidants.

An increase of antioxidants in diet provides one possible solution to excessive ROS production. Some *in vitro* and epidemiological studies indicate the association between a high intake of antioxidant compounds and a reduced risk of cardiovascular and cancer diseases (PRIOR [1], STANNER & al. [2], GOSZCZ & al. [3]). Fresh fruits and vegetables are excellent sources of phytochemicals with antioxidant properties. Health benefits of plant bioactives are often linked to the interactive and synergistic effects of the wide range of chemical entities highly correlated with each other.

Within the *Brassicaceae* family, red cabbage (*Brasica oleracea* var. *capitata* f. *rubra*) represents a dietary source rich in phytochemicals, in particular (poly)phenolics, flavonoids, anthocyanins, ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene, lutein, etc. (JAGDISH SINGH & al. [4]). Anthocyanins are water-soluble vacuolar pigments with proved health-promoting properties mainly based on their strong antioxidant activity (HE & GIUSTI [5]). The mechanism by which (poly)phenolics act as antioxidants is likely to involve: (i) a direct antioxidant activity through interaction with ROS and free radicals resulted from biomolecules; (ii) chelating metal ions (Fenton chemistry); (iii) the inactivation of enzymes responsible for superoxide radical generation (xanthine oxidase, protein kinase C); (iv) the activation of antioxidant enzymes (superoxide dismutase, glutathione *S*-transferase, glutathione peroxidase, catalase) [3]. Emerging theories from the current studies show that the (poly)phenolics molecular mechanism may be linked to the modulation of gene expression (RAY & al. [6]). These studies suggest that the modulation of genes such as those related to *in vivo* inflammation pathways or genes associated with the antioxidant activity of hydroxytyrosol, seems to be the molecular target for the antioxidant activity of polyphenolics, in particular from olives.

Epidemiological studies indicate that a high intake of *Brassica* vegetables may lead to a reduced risk of cardiovascular and cancer diseases (KRISTAL & LAMPE [7]). Other studies have reported hypocholesterolaemic (KOMATSU & al. [8]), hepatoprotective (SINGAB & al. [9]), neuroprotective (HEO & LEE [10]) and nephroprotective (KATAYA & HAMZA [11]) effects of red cabbage extracts in rats, in particular due to phenolics/anthocyanins content.

There are very few studies showing the antioxidant effects of red cabbage in erythrocytes against free-radical induced oxidative haemolysis. Thus, the aim of the present work was to evaluate and compare the content of antioxidant compounds with phenolic structure extracted from several red cabbage samples, and to determine their *in vitro* antioxidant and antihaemolytic activities.

## 2. Materials and Methods

### 2.1. Preparation of samples

Seven commercial samples of red cabbage (*Brassica oleracea* var. capitata f rubra) purchased in May 2015 from different local markets, three of which originated from Romania, two from Poland, one from Holland and one from Germany, were used for the present study. Cut slices of samples were freeze-dried using a lyophilizer (Alpha 1-4 LD plus, CHRIST) at -45°C. The freeze-dried cabbage was powdered using the knife mill (Grindomix GM 200, RETSCH). Chemical reagents of analytical grade without further purification were used.

## **2.2. Extraction procedures**

Extraction of bioactive antioxidant compounds (anthocyanins, phenolics, flavonoids) from lyophilized red cabbage was conducted using two solvent systems: (i) 70% (v/v) ethanol in water; (ii) acidified 70% (v/v) ethanol 0.05% HCl, for one hour at 4 °C. The obtained mixtures were centrifuged at 8000 rpm for 10 minutes at 4 °C. The 320R refrigerated centrifuge (HETTICH) was used.

## **2.3. Physico-chemical characterization**

Two quality parameters of the lyophilized red cabbage samples were measured, as follow: (i) water activity using the AW Meter LabMaster-aw (NOVASINA); (ii) moisture content determined at 105 °C using the moisture analyzer (ML-50, A&D Co. Ltd.). In addition, pH of the prepared extracts was measured using the Orion 2-star pH meter (THERMO SCIENTIFIC).

## **2.4. Total anthocyanins**

The content of total anthocyanins was determined spectrophotometrically by the pH differential method (GIUSTI & WROLSTAD [12]). The Specord 200Plus UV-Vis spectrophotometer (ANALYTIK JENA) was used. The content was expressed as milligram cyanidin-3-O-glucoside (Cyn-3-O-G) equivalents per 100 g dry mass (mg 100g<sup>-1</sup> DM).

## **2.5. Total flavonoids**

The content of total flavonoids was determined using the aluminium chloride colorimetric method (KUMAR & al. [13]). The content was expressed as milligram quercetin equivalents per 100 g dry mass (mg 100g<sup>-1</sup> DM).

## **2.6. Total phenolics**

The content of total phenolics was determined according to the Folin-Ciocalteu spectrophotometric method (SINGLETON & ROSSI [14]). The content was expressed in milligram of gallic acid equivalents per 100 g dry mass (mg GAE 100g<sup>-1</sup> DM).

## **2.7. Antioxidant activity by ferric reducing antioxidant power (FRAP)**

The total antioxidant capacity was determined by the ferric reducing ability assay described by Benzie and Strain (BENZIE & STRAIN [15]). The results were expressed as milligram ascorbic acid per 100 g dry mass (mg 100g<sup>-1</sup> DM).

## **2.8. Inhibitory effects on AAPH-induced hemolysis**

The antihaemolytic activity of red cabbage extracts in 70% (v/v) ethanol was determined using the method described by Asgari et al (ASGARY & al. [16]). Sheep red blood cells (RBC) were separated by centrifugation and washed with PBS buffer pH 7.4 until the supernatant was colorless. A 7% suspension of RBC was prepared in PBS buffer and used for incubation at 37 °C for 2 hours in the presence of 2,2'-azo-bis-(2-amidinopropane) dihydrochloride (AAPH) (25 mM) and red cabbage extracts. Control sample without extracts was used. The inhibition of haemolysis was determined by measuring the absorption at 540 nm. It was calculated accordingly as percentage of inhibition (%).

## **2.9. Statistical analysis**

All analyses were made in duplicate ( $n=2$ ). Data are expressed as mean  $\pm$  standard deviation from two parallel measurements. The processing of data was performed using mathematical and

statistical methods with “IBM SPSS 21.0” software, following hypothesis testing and correlation between variables by calculating the Pearson’s correlation coefficient, at a significance level of risk  $\alpha \leq 5\%$  and probability  $P \geq 95\%$ .

### 3. Results and Discussions

#### Physical-chemical characterization of red cabbage powders/extracts

Seven commercial samples of red cabbage (*Brassica oleracea* var. capitata f rubra) of different geographical origin purchased from local markets were used in the present investigation, as follow: **1** (Romania), **2** (Romania), **3** (Romania), **4** (Poland), **5** (Poland), **6** (Holland) and **7** (Germany).

Extraction was performed by conventional extractive technology using 70% ethanol in water (v/v) as environmental friendly solvent. Because low amounts of acids facilitate extraction of polyphenolic-based compounds, acidified ethanol with 0.05% HCl was also investigated for the extraction of investigated antioxidant compounds. All extraction runs were conducted at 4 °C in order to minimize degradation of bioactives, in particular anthocyanins. We further used these two types of extracts to determine the content of anthocyanins, flavonoids, phenolics and antioxidant activity despite that other solvents such as methanol might have been better extracted particular compounds.

Some of the physical-chemical attributes of the lyophilized samples and extracts are given in Table 1. The moisture values of a sample indicates its total content of water, while the water activity values reveal the availability of water for biological processes, including microbial contamination. These two characteristics are often used to describe the food safety and prediction of products’ shelf life. By measuring the pH of the obtained hydroethanolic extracts, in accordance to Romanian Pharmacopoeia (FARMACOPEEA ROMANA [17]), we found that they are neutral, while the acidified hydroethanolic extracts are weak acids, as shown in Table 1.

**Table 1.** Physical-chemical characteristics of red cabbage samples of different origin.

<i>Brassica</i> samples	Moisture of lyophilized sample (%)	Water activity (aw) of lyophilized sample at 25 °C	pH of extract	
			70% EtOH	0.05% HCl in 70% EtOH
<b>1</b>	5.915	0.282	6.489	5.457
<b>2</b>	9.709	0.454	6.623	5.660
<b>3</b>	4.460	0.208	6.585	5.820
<b>4</b>	7.260	0.382	6.624	5.591
<b>5</b>	7.563	0.358	6.600	5.720
<b>6</b>	4.000	0.150	6.378	5.630
<b>7</b>	7.335	0.365	6.505	5.513

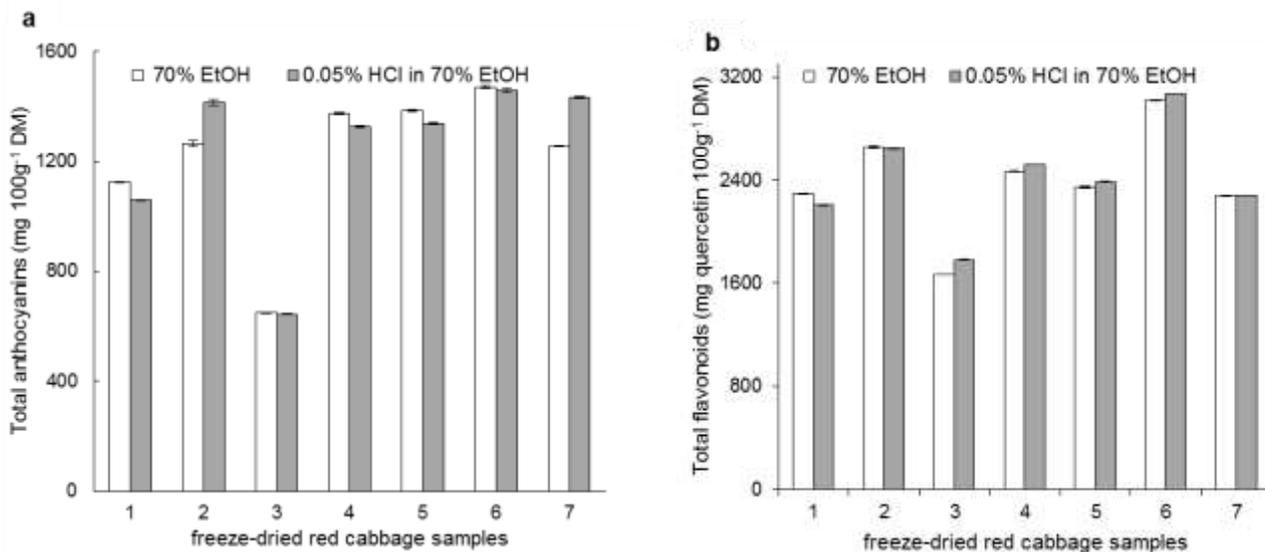
#### Antioxidant compounds and activity of red cabbage crude extracts

Based on the significant contribution of red cabbage consumption to human health, hydroethanolic (70% EtOH) and acidified hydroethanolic (0.05% HCl in 70% EtOH) extracts were analysed in terms of anthocyanins and flavonoids contents, and of total antioxidant activity,

as measured by Folin-Ciocalteu and FRAP assays. For comparison reasons, all values were calculated on percentage and dry mass (DM) basis.

No statistically significant differences ( $P>0.05$ ) were found between the content of the antioxidant compounds and the extraction solvent used in this work.

The content of total anthocyanins and flavonoids in *Brassica oleracea* extracts is presented in Fig. 1.



**Figure 1.** The content of total anthocyanins (a) and flavonoids (b) in two different extracts of red cabbage; vertical bars indicate SE of the means.

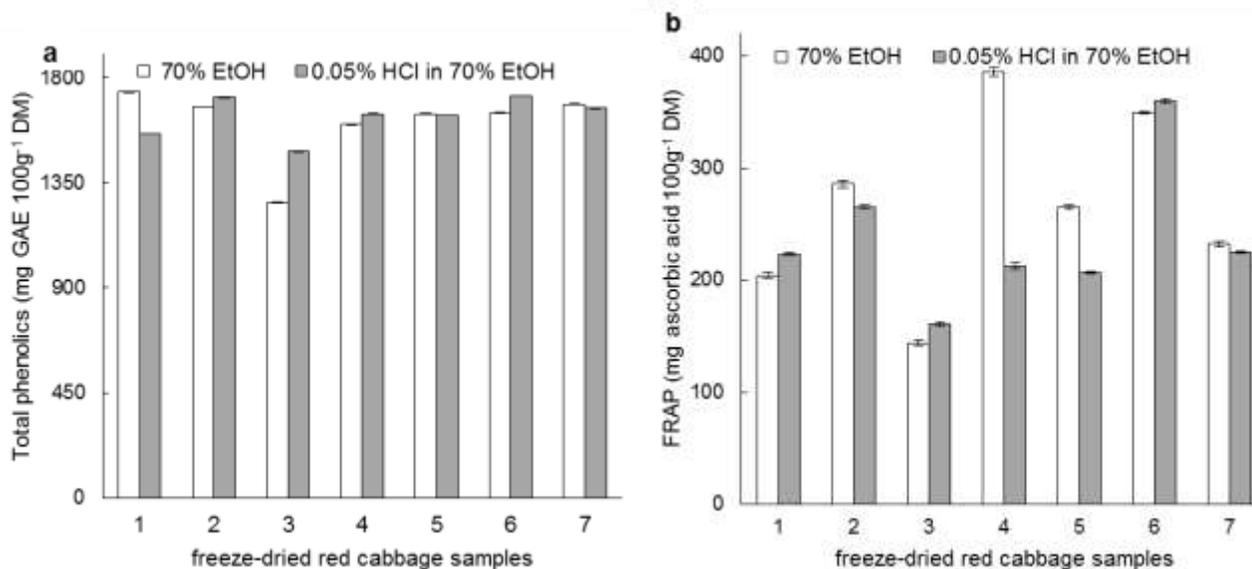
The mean values of anthocyanins content in the investigated samples using two extraction solvent systems varied between 648.83 and 1471.54 mg 100g<sup>-1</sup> DM, depending on the varieties originated from different regions. The statistical analysis showed that despite the fact that low amounts of acids added to the extraction solvent gave better extraction of anthocyanins, the differences were not significant. The highest concentration was obtained from sample 6 (red cabbage from Holland) regardless the applied extraction solvent systems. The anthocyanins level in samples grown in Romania varied between 648.83 and 1265.76 mg 100g<sup>-1</sup> DM, most probably linked to different varieties and cultivation regions. Other reported values of anthocyanins concentration determined by the same pH differential spectrophotometric method, and on a dry mass basis, were similar and varied between 10-19 g kg<sup>-1</sup> DM for the “Gradur” and “Roxy” hybrids from North-Central Italy (PICCAGLIA & al. [18]). The content of anthocyanins in red cabbage is influenced by several factors, such as genotypes and environmental conditions. The same authors have found that red cabbage variety seems to be a major factor compared to fertilization practises.

The results regarding the total flavonoids content indicate variations between 1727.36 and 3047.53 mg quercetin equivalents 100g<sup>-1</sup> DM in the seven investigated samples, with the highest amount for sample 6 regardless the applied type of extraction solvent systems. The statistical analysis showed that despite the fact that low amounts of acids added to extraction solvent gave better extraction of flavonoids, the differences were not significant. Most previous studies on red cabbage reported the content of total flavonoids expressed on a fresh mass basis, and different equivalents (catechin, quercetin, rutin), so that comparison with our data is not appropriate to

perform, but a recent study on secondary metabolites from red cabbage (variety or region not specified) reported similar flavonoids content in methanolic extracts using the same analytical procedure (17.44 mg quercetin equivalents  $g^{-1}$  DM) (GAAFAR & al. [19]).

The mean value of total anthocyanins of investigated ethanolic and acidified ethanolic extracts was 1228.81 mg  $100g^{-1}$  DM, while of total flavonoids was 2402.27 mg quercetin  $100g^{-1}$  DM.

Polyphenolic compounds are antioxidant molecules which can interact with both radical and non-radical ROS, as well as with reactive species produced from cell molecules (QUIDEAU & al. [20]). The antioxidant properties of such compounds are considered to be responsible for their health benefits. Different investigative methodologies are used for this purpose, such as oxygen radical absorbance capacity assay (ORAC), total reactive antioxidant potential (TRAP), ferric-reducing antioxidant power assay (FRAP), Trolox equivalent antioxidant capacity (TEAC), total phenolics assay by Folin-Ciocalteu, thiobarbituric acid reactive substances assay (TBARS), DPPH radical scavenging activity or  $\beta$  carotene-linoleic acid assay. In our work, we used the FRAP and Folin-Ciocalteu assays to evaluate the total antioxidant activity of the hydrophilic red cabbage extracts. The results are presented in Fig. 2.



**Figure 2.** The content of total phenolics (a) and the antioxidant activity measured by FRAP assay (b) in two different extracts of red cabbage; vertical bars indicate SE of the means.

With the exception of sample **3**, the others showed very similar total phenolics concentration (mean values between 1621.29 and 1693.84 mg GAE  $100g^{-1}$  DM for both type of extracts). Our results showed that using 70% ethanol as extraction solvent proved better extractability of phenolics for sample **1**, while acidified ethanol determined extraction of higher amounts of phenolics in sample **6**. The statistical analysis showed that despite the fact that low amounts of acids added to ethanol improved the extraction of phenolics, the differences were not significant. Higher amounts of phenolics in lyophilized red cabbages using the same Folin-Ciocalteu procedure have been reported by other authors, but on hydromethanolic extracts (GAAFAR & al. [19], DUCHNOWICZ & al. [21]), while others reported much lower phenolics content in the Romanian red cabbage cv. CB10089 (VICAS & al. [22]).

The mean values of FRAP of 70% hydroethanolic extracts varied between 141.45 mg ascorbic acid 100g<sup>-1</sup> DM (sample 3) and 389.84 mg ascorbic acid 100g<sup>-1</sup> DM (sample 4). The mean values of FRAP of 0.05% HCl acidified 70% hydroethanolic extracts varied between 159.28 mg ascorbic acid 100g<sup>-1</sup> DM (sample 3) and 357.67 mg ascorbic acid 100g<sup>-1</sup> DM (sample 6). As noticed in Fig. 2, sample 4 showed very high antioxidant activity compared to the other analysed samples.

Comparison of our results on the antioxidant activity of red cabbage hydroethanolic extracts with previous published ones is a difficult task, as different extraction procedures and methods of analysis of both water- and lipid-soluble antioxidants have been employed. However, using both phenolics and FRAP measurements, our results confirmed that the extract from sample 6 showed the highest value of total antioxidant activity, while the extract from sample 3 showed the lowest one. These results are in accordance to those regarding the determined content of anthocyanins and flavonoids.

The results of statistical correlation by regression analysis indicate a positive correlation of anthocyanins with flavonoids ( $R^2=0.843$ ,  $P<0.01$ ). The total antioxidant activity positively correlates with anthocyanins and flavonoids, using both methods of measurements (Folin-Ciocalteu and FRAP). Good positive correlations were found between FRAP and flavonoids ( $R^2=0.817$ ,  $P<0.01$ ) and between phenolics and anthocyanins ( $R^2=0.807$ ,  $P<0.01$ ), respectively. Other authors have also found that flavonoids, anthocyanins and ascorbic acid are the best predictors for the antioxidant activity by FRAP of cabbage (KAULMANN & al. [23]).

This study affirmed that the level of polyphenolic-based antioxidant compounds of red cabbage varied extensively according to genetic and environmental conditions. Also, we do not exclude the influence of the period of commercial storage on the content of antioxidant compounds and activity.

#### **Antihaemolytic activity of red cabbage hydroethanolic extracts**

The oxidative damage induced by ROS can be investigated *in vitro* using erythrocytes as model of biomembranes as they may be disrupted in the presence of the reactive species causing haemolysis (CLEMENS & al. [24]).

Because erythrocytes are often used for *in vitro* studies regarding the nutritional impact on disorders induced by oxidative stress, in the present study we investigated the potential of red cabbage crude extracts to inhibit such oxidative damage. Erythrocytes are known for their high sensitivity to oxidative damage mainly because of the content of membrane proteins and polyunsaturated fatty acids (CHIU & al. [25]).

We used the water soluble azo compound AAPH as free peroxy radical generator in erythrocytes, at 37 °C. Control samples consisting of erythrocytes and extracts incubated alone without AAPH were also prepared, suggesting the absence of abnormal oxidation. We recorded a significant inhibition of AAPH-induced oxidative haemolysis of erythrocytes in the presence of 70% hydroethanolic extracts of red cabbage after 2 hours of incubation, as presented in Table 2. Therefore, bioactive compounds (flavonoids, anthocyanins) from red cabbage may quench the chain propagating peroxy radicals generated by AAPH in erythrocytes, thus decreasing the extent of peroxidation and diminishing haemolysis. As noticed in Table 2, the efficiency of the antihemolytic activity varies in the order 4>1>6>2>3>5>7.

The most effective inhibition was produced by extract 4, which also demonstrated very high antioxidant activity by FRAP (Fig. 2) compared to the other samples. Interestingly, sample 6 which showed mean values of bioactive compounds higher than the other investigated samples

indicated lower antihaemolytic activity. This may be related to the contribution of other important water soluble bioactive compounds existing in the red cabbage hydrophilic extracts, such as ascorbic acid, glucosinolates, minerals which may act synergistically and could explain the mechanism of interaction with erythrocytes membrane. In a previous study it was shown that chain-breaking ascorbic acid suppressed the haemolysis dose dependently (NIKI & al. [26]). We found that weak or no statistical correlations exist between the antihaemolytic activity and the content of bioactive compounds in the investigated samples.

**Table 2** The haemolytic inhibition of red cabbage hydroethanolic extracts of different origins; mean values  $\pm$  standard deviation.

<b>Brassica samples</b>	<b>Inhibition of haemolysis (%)</b>
<b>1</b>	74.91 $\pm$ 0.37
<b>2</b>	44.42 $\pm$ 1.06
<b>3</b>	43.72 $\pm$ 0.51
<b>4</b>	93.49 $\pm$ 0.09
<b>5</b>	30.78 $\pm$ 0.56
<b>6</b>	47.64 $\pm$ 0.61
<b>7</b>	5.35 $\pm$ 0.88

Considering all samples, we found a mean value of 48.61 % haemolytic inhibition caused by the different red cabbage extracts, indicating their protective effects against erythrocytes oxidative damage most probably through the inhibition of lipid peroxidation. As far as we know, no other similar haemolysis studies have been reported on red cabbage, but there are studies reporting: (i) the cholesterol decrease in blood samples from patients diagnosed with hypercholesterolemia caused by red cabbage extract added to collected erythrocytes without affecting membrane fluidity (DUCHNOWICZ & al. [21]); (ii) the inhibitory effect of red cabbage extract on peroxynitrite-induced oxidation in human erythrocytes, by measurement of nitrated tyrosine, protein carbonylation and lipid peroxidation (KOŁODZIEJCZYK & al. [27]); (iii) the protective role of flavonoids, individual or in natural extracts, against damages in erythrocytes in a concentration and time dependent pattern (ASGARY & al. [16], SULAIMAN & al. [28]). Antioxidant compounds, in particular flavonoids, have been shown to exert their biological/pharmacological properties through cell membrane interactions (ERLEJMAN & al. [29]). Using electron spin resonance technique (ESR) and erythrocytes model, a Polish research group showed that the flavonoid quercetin localizes at the polar-apolar interface of the lipid bilayer (PAWLIKOWSKA-PAWLÉGA & al. [30]). Such distribution may induce alteration of erythrocytes membrane permeability, thus protecting against peroxidation. Low concentrations of quercetin did not induce haemolysis, but higher levels lead to modifications of erythrocytes shape. It is considered that lipophilicity/chemical structure of the flavonoid molecules highly influences the flavonoid-lipid interactions.

#### 4. Conclusions

The content of anthocyanins, flavonoids, phenolics, as well as *in vitro* antioxidant and antihaemolytic activity in seven samples of red cabbages were determined and compared.

The present study indicates that the level of polyphenolic-based antioxidant compounds varies according to genetic and environmental conditions of investigated samples. Statistically,

we found that low acidification of ethanol solution used for extraction does not significantly influence the recovery of such bioactives. The mean value of total anthocyanins of ethanolic and acidified ethanolic extracts was 1228.81 mg 100g<sup>-1</sup> DM while of total flavonoids content was 2402.27 mg quercetin 100g<sup>-1</sup> DM. We found a positive correlation between anthocyanins and flavonoids. We report high total antioxidant activity of crude extracts as measured by Folin-Ciocalteu (1620.35 mg GAE 100g<sup>-1</sup> DM) and FRAP (251.39 mg ascorbic acid 100g<sup>-1</sup> DM) assays. The total antioxidant activity positively correlates with anthocyanins and flavonoids, using both methods of measurements.

In addition, the cytoprotective effects of the different ethanolic crude extracts from red cabbage against AAPH-induced oxidative damage in erythrocytes further indicates the beneficial role of red cabbage in preventing oxidative stress.

Thus, our studies demonstrate the potential health benefits of red cabbage extracts based on the high content and *in vitro* activity of polyphenolic antioxidant compounds, and provides the first scientific evidence for their protective role against cytotoxic effects of reactive oxygen species in erythrocytes.

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