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## Research Article

# Erythrocyte Antioxidant Protection of Rose Hips (*Rosa* spp.)

C. Widén,<sup>1,2</sup> A. Ekholm,<sup>1</sup> M. D. Coleman,<sup>3</sup> S. Renvert,<sup>2</sup> and K. Rumpunen<sup>1</sup>

<sup>1</sup> Department of Plant Breeding and Biotechnology Balsgård, Swedish University of Agricultural Sciences, Fjälkestadvägen 459, 291 94 Kristianstad, Sweden

<sup>2</sup> School of Health and Society, Kristianstad University, 291 88 Kristianstad, Sweden

<sup>3</sup> School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK

Correspondence should be addressed to C. Widén, [cecilia.widen@hkr.se](mailto:cecilia.widen@hkr.se)

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Rose hips are popular in health promoting products as the fruits contain high content of bioactive compounds. The aim of this study was to investigate whether health benefits are attributable to ascorbic acid, phenols, or other rose-hip-derived compounds. Freeze-dried powder of rose hips was preextracted with metaphosphoric acid and the sample was then sequentially eluted on a C<sub>18</sub> column. The degree of amelioration of oxidative damage was determined in an erythrocyte *in vitro* bioassay by comparing the effects of a reducing agent on erythrocytes alone or on erythrocytes pretreated with berry extracts. The maximum protection against oxidative stress, 59.4 ± 4.0% (mean ± standard deviation), was achieved when incubating the cells with the first eluted meta-phosphoric extract. Removal of ascorbic acid from this extract increased the protection against oxidative stress to 67.9 ± 1.9%. The protection from the 20% and 100% methanol extracts was 20.8 ± 8.2% and 5.0 ± 3.2%, respectively. Antioxidant uptake was confirmed by measurement of catechin by HPLC-ESI-MS in the 20% methanol extract. The fact that all sequentially eluted extracts studied contributed to protective effects on the erythrocytes indicates that rose hips contain a promising level of clinically relevant antioxidant protection.

## 1. Introduction

Oxidative stress is associated with many different diseases such as heart- and cardiovascular disease, diabetes, obesity, and cancer. Epidemiological studies show that diets rich in fruits promote good health, at least partly through delaying the onset of diseases associated with oxidative stress [1]. These beneficial effects may be mediated by different phytochemicals with high antioxidant capacity, of which the polyphenols is a large group abundantly found in berries [2, 3]. Measurement of antioxidant capacity can be performed with many different methods. The relevance of general, chemical methods and their relationship to actual human health benefits is, however, controversial [4–6]. Human cell-based systems may provide more biological relevance than simple chemical assessments and they also allow the opportunity to consider interactions between added nutrients and functionally complete cellular enzyme systems, as well as with the intact membranes of living cells.

Erythrocytes can serve as a relevant human cell model in the investigation of bioavailability and antioxidant protection by natural products against oxidative stress. The antioxidant potential of plant phytochemicals against oxidative stress has previously been assessed using different erythrocyte methods. The degree of lipid peroxidation of the cell membrane has been investigated, using either intact erythrocytes or erythrocyte membranes for measuring malondialdehyde, an indicator of lipid peroxidation [7, 8]. Coleman [9] used methaemoglobin generation as a model for oxidative stress. Other laboratories have focused on the levels of redox enzymes [10–13] as well as the use of free radical generators to induce erythrocyte lysis [12, 14, 15]. Strategies for the study of dietary antioxidant protection using erythrocytes have also been published [16–19]. Erythrocytes have been used to test for oxidative stress in several different diseases [20–22] and for *in vitro* testing of cell-permeating therapeutic antioxidants. Honzel et al. [23] and Blasa et al. [24] have developed erythrocyte models

in order to identify natural products, which may provide sustained intracellular protection from oxidative damage.

The suitability of the erythrocyte as an assay for oxidative stress measurements lies in its adaptation to the prevention of oxidative stress-mediated perturbation of the structure and therefore the function of haemoglobin. Erythrocytes contain over 90% of their weight as haemoglobin, and the cells stabilize this protein with the second highest intracellular GSH level (after the liver) in the body [9]. The high GSH concentration quenches reactive oxygen species-mediated structural haemoglobin damage, which can occur as a result of superoxide formation, which in turn arises from normal oxygen carriage [9]. The GSH maintenance system also provides the reducing power to maintain other cellular antioxidants in their reduced states. Various phytochemicals with different chemical properties may combat oxidative stress within the erythrocyte and potentially preserve intracellular thiols [9].

There is a growing interest in fruits and berries as rich sources of many bioactive compounds that may promote health. Many studies have investigated artificial substances and/or pure compounds in high concentrations rather than the joint effect of phytochemicals in complex foods. During the last decade, rose hips have gained increased interest since they contain compounds that are known to possess several antioxidant, antimutagenic, and anticarcinogenic effects [25, 26]. Rose hips have been used to treat conditions such as arthritis [27–29], rheumatism, and diabetes. The major bioactive compounds within rose hips are phenols, ascorbic acid, tocopherols,  $\beta$ -carotene, lycopene, tannins, pectins, sugars, organic acids, amino acids and essential fatty acids [30, 31]. Other rose-hip-derived compounds reported include galactolipids [31] and triterpenic acids [32]. Interestingly, the antioxidant effects of these compounds cannot fully account for the clinical effects of rose hip powder [33] and the efficacy of the phenolic compounds in the rose hips has yet to be evaluated in controlled clinical trials [30]. Indeed, it still remains unclear whether the protective effects of polyphenols to human health are attributable to the phenols or other agents in the diet [30], such as phytochemicals, that may enhance total oxidant-scavenging capacities by binding to erythrocytes [34]. The main purpose of this study was to investigate different aspects of the antioxidant protection of rose hips on oxidative stress in an erythrocyte *in vitro* test system.

## 2. Materials and Methods

**2.1. Chemicals and Cells.** Methanol, formic acid, acetonitrile, 85% orthophosphoric acid, and meta-phosphoric acid were obtained from Merck (Darmstadt, Germany). Ascorbic acid, ascorbate oxidase, dimethyl sulfoxide (DMSO),  $\text{KH}_2\text{PO}_4$ ,  $\text{Na}_2\text{PO}_4$ , EDTA, and  $\text{H}_2\text{O}_2$  were purchased from Sigma-Aldrich (Seelze, Germany). Phosphate-buffered saline (PBS), without calcium or magnesium, and dichlorofluorescein diacetate (DCF-DA) were obtained from Invitrogen (Lidingo, Sweden). The standards used for HPLC-ESI-MS

analysis (catechin, proanthocyanidin, rutin, quercetin galactoside, cyanidin glucoside) were purchased from Extrasynthese (Genay, France).

**2.2. Plant Materials.** To evaluate the antioxidant uptake in erythrocytes rose hips from three advanced selections (“BRo30173,” “BRo30289,” and “BRo05035”) were sampled at full maturity. The seeds were removed and the remaining flesh with skin was lyophilized and ground to a fine powder in a laboratory mill (Yellow line, A10, IKA-Werke, Staufen, Germany) before extraction.

**2.3. Preparation of Polyphenol Rich Extracts.** The freeze-dried powders of rose hips from the three selections were blended in equal proportions and 2.5 g of the powder was added to 50 mM metaphosphoric acid (50 mL) for preextraction. The rose hip preextract (PE) was kept in an ultrasonic bath for 15 min before centrifugation at 4500 rpm for 10 min. The supernatant was applied to a  $\text{C}_{18}$  (EC) column (Isolute SPE Columns, Biotage, Sorbent AB) that had been pre-equilibrated with 100% methanol and washed with  $\text{dH}_2\text{O}$ . A sequential elution was performed and the first obtained extract (E1A) consisted of the metaphosphoric acid eluent of the pre-extract. Extract two (E2A) and three (E3A) consisted of the eluents with 50 mL 20% aqueous methanol and 50 mL 100% methanol, respectively. Metaphosphoric acid and methanol were used as they preferentially extract different bioactive compounds according to their physicochemical properties. The solvents of the extracts were removed using a rotary evaporator at 45°C. The concentrated extracts were then dissolved in 50 mM metaphosphoric acid.

**2.4. Enzymatic Removal of Ascorbic Acid.** Ascorbate oxidase (AO, Sigma Aldrich) was used for enzymatic removal of ascorbic acid by reducing ascorbic acid to dehydroascorbic acid. Ascorbate oxidase was dissolved in a phosphate buffer consisting of 100 mM  $\text{KH}_2\text{PO}_4$ , 4 mM  $\text{Na}_2\text{PO}_4$ , and 5 mM EDTA, and pH was set to 5.6. Aliquots of the extracts (E1A–E3A) were taken, and pH adjusted to 5.6. These extracts were then treated with ascorbate oxidase to provide ascorbate-depleted extracts (E1B–E3B). For this purpose 100 units of ascorbate oxidase was added to the test tubes containing the samples and left in the dark at room temperature for 24 hours.

**2.5. Analysis of Ascorbic Acid Content in the Extracts.** The ascorbic acid content of the extract was determined on a Shimadzu HPLC system (SIL-10A autosampler, SCL-10AVP control unit, LC-10AD pump, SPD-10AV VP UV-Vis detector unit, BergmanLabora, Sweden). The pre-extract (PE) and the metaphosphoric acid extract (E1A) were diluted with 2% meta-phosphoric acid, 20 and 30, times respectively, before analysis. No dilution was performed with the other extracts. The isocratic eluent consisted of 0.05M  $\text{NaH}_2\text{PO}_4$  and orthophosphoric acid (8.5%), pH of the eluent was adjusted to 2.8. The separation was performed using a Restek, 150 × 4.6 mm, column kept at 30°C (Column Chiller, Sorbent AB) and a guard column. Detection was carried

out at 254 nm. Evaluation of data was done with Shimadzu Class-VP software (version 6.13 SP2) using retention times and spectral data as compared with an external standard of ascorbic acid. Each sample was analyzed in triplicate.

**2.6. Analysis of Phenols by HPLC-ESI-MS.** The content of major single phenols in the different extracts was measured on a Perkin Elmer Sciex API 150EX Single Quadrupole mass spectrometer (with a Turbo Ion Spray interface) according to a modified method from that described by Salminen et al. [35]. The HPLC system consisted of two Perkin Elmer pumps connected to a Perkin Elmer autosampler (Serie 200). The compounds were separated using a Phenomenex Synergi 4 $\mu$  Hydro-RP 80A, 250  $\times$  4.6 mm column, and a C<sub>18</sub> precolumn. The mobile phase consisted of 0.4% formic acid (Buffer A) and acetonitrile (Buffer B). The eluent was run at a flow rate of 1.0 mL min<sup>-1</sup> and the gradient elution was as follows: 0% B (0–3 min), 30% B (3–30 min), 40% B (30–35 min), 40% B (35–38 min), and 0% B (38–42.5 min). The injection volume was for all samples 8  $\mu$ L. The eluent was split to 0.3 mL min<sup>-1</sup> before being introduced to the ESI-MS system. The electrospray chamber was operated at 4.0 kV in the negative ion mode and mass ions obtained by acquiring data in peak jump and scan mode. Catechin was detected at m/z 289.3 (M-H)<sup>-</sup>, proanthocyanidin monomer 577.5 (M-H)<sup>-</sup>, proanthocyanidin dimer 577.5 (M-H)<sup>-</sup>, rutin 609.5 (M-H)<sup>-</sup>, quercetin galactoside 463.4 (M-H)<sup>-</sup>, quercetin glucoside 463.4 (M-H)<sup>-</sup>, and cyanidin glucoside 477.1 (M-H)<sup>-</sup>. The general conditions for the quantitative HPLC-ESI-MS analyses were the following: nebulizer gas 9.0 L min<sup>-1</sup>, curtain gas 12.0 L min<sup>-1</sup>, and dry gas temperature 300°C.

**2.7. Determination of Total Phenols.** The content of total phenols was measured using the Folin-Ciocalteu method [36]. In brief, five  $\mu$ L of the different extracts were mixed with 100  $\mu$ L 5% ethanol, 200  $\mu$ L Folin-Ciocalteu reagent, 2 mL of 15% Na<sub>2</sub>CO<sub>3</sub>, and 1 mL of dH<sub>2</sub>O. The absorbance was measured at 765 nm after 2 h incubation at room temperature. Gallic acid was used as a standard and the total content of phenols was expressed as mg gallic acid equivalents (GAE)/g dry weight (dw).

**2.8. Ferric Reducing Ability of Plasma.** The ferric reducing ability of plasma (FRAP) of the extracts was measured according to the method developed by Benzie and Strain [37], but modified to fit a 96-well format [38]. The different extracts were diluted 100-fold. Ten  $\mu$ L of these preparations were incubated at 37°C and then mixed with 260  $\mu$ L of ferric-TPTZ reagent (prepared by mixing 300 mM acetate buffer, pH 3.6; 10 mM of 2,4,6-tripyridyl-s-triazine in 40 mM HCl; and 20 mM FeCl<sub>3</sub> in the ratio of 4:1:1; the solution was kept at 37°C). The absorbance was measured at 595 nm after 4 min on a plate reader (Sunrise, Tecan Nordic AB, Sweden). Fe<sup>2+</sup> was used as a standard and L-ascorbic acid was used as a control where one mole of ascorbic acid corresponds approximately to two moles of FRAP (we obtained and used the value 2.02).

**2.9. Preparation of Erythrocytes.** The preparation of erythrocytes was performed as described by Honzel et al. [23]. Briefly, a healthy human volunteer served as blood donor. Peripheral venous blood samples were drawn into sodium K<sub>2</sub>-EDTA vials (BD Vacutainer, UK). The vials were centrifuged for 5 min at 2400 rpm. Plasma and leukocytes were removed and the erythrocytes were harvested by pipettes and transferred into new vials. The erythrocytes were washed three times with PBS in a centrifuge at 2400 rpm for 5 min.

**2.10. Analysis of Antioxidant Protection in the Erythrocyte Model.** The protocol for the erythrocyte bioassay was based on the cellular antioxidant protection assay using erythrocytes (CAP-e) [23] modified to a microplate-based assay, but using H<sub>2</sub>O<sub>2</sub>, as the free radical generator. Briefly, from the remaining packed erythrocytes, 0.12 mL was added to 12 mL of PBS. The erythrocytes were then treated with serial dilutions of the previously obtained extracts (PE, E1A–E3A and E1B–E3B). The erythrocyte suspension was incubated in the dark on a rocker at room temperature for 120 min. The erythrocytes were then washed twice in PBS and any extracellular potential antioxidants were thereby removed. The cell pellet was then lysed through the addition of dH<sub>2</sub>O and the lysed sample treated with the fluorescent dye 5-(and-6)-carboxy-2',7'-dichlorofluorescein (DCF-DA), which becomes fluorescent as a result of oxidative damage. After this the sample was exposed to free radicals by addition of 167 mM hydrogen peroxide (hydroxyl free radical generator). The degree of antioxidant damage was recorded after 10 min by measuring the fluorescence intensity of each sample. The mean DCF-DA fluorescence intensity was compared between untreated erythrocytes (negative controls), hydrogen-peroxide-treated erythrocytes (positive controls), and erythrocytes pretreated with extracts from rose hips on three separate plates.

**2.11. Analysis of Erythrocyte Uptake of Phenols.** From the remaining packed erythrocytes, pure erythrocytes were treated with extract E2A. All measurements were performed with three true replicates. For this purpose, the erythrocyte cell suspension (1/3 sample and 2/3 purified erythrocytes) was incubated in the dark on a rocker at room temperature for 60 min. The erythrocytes were washed twice in PBS to remove the antioxidants not able to enter the cells after the 60 min incubation. The supernatants following the wash were retained for analysis. The cell pellet was lysed with absolute ethanol, vortexed and placed in an ultrasonic bath for 5 min, and then centrifuged at 13000 rpm for 5 min. The supernatant was then kept for analysis using HPLC-ESI-MS as described in Section 2.6.

**2.12. Statistical Analysis.** The results of the oxidative stress experiments were expressed as the mean  $\pm$  standard deviation. Each observation was repeated in triplicate in different 96-well plates. Statistical analyses were carried out using the Minitab 16 software (Minitab, State College, PA, USA). Paired *t*-test analyses were performed to reveal any significant difference between treatments (extracts).

TABLE 1: Content of ascorbic acid and its contribution to antioxidant capacity of different extracts (E1–E3, without (A) or with (B) ascorbate oxidase treatment) obtained from sequential elution of a metaphosphoric preextract (PE) of rose hips. Data are presented as mg ascorbic acid/g dw rose hip powder, mg ascorbic acid/mL extract, and FRAP antioxidant capacity mmol Fe<sup>2+</sup>/L extract (mean ± standard deviation).

Fraction	Ascorbic acid (mg/g dw)		Ascorbic acid (mg/mL)		FRAP ascorbic acid (mmol Fe <sup>2+</sup> /L)	
	A	B	A	B	A	B
PE (HPO <sub>3</sub> )	80.18 ± 1.02		2.74 ± 0.05		31.40 ± 0.62	
E1 (Eluent of PE)	42.03 ± 1.60	0.03 ± 0.06	5.97 ± 0.23	0.00 ± 0.01	68.41 ± 2.60	0.06 ± 0.09
E2 (20% MeOH)	1.41 ± 1.72	0.00 ± 0.00	0.59 ± 0.72	0.00 ± 0.00	6.72 ± 8.23	0.00 ± 0.00
E3 (100% MeOH)	0.04 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.21 ± 0.05	0.00 ± 0.00

### 3. Results

In this study, the protection against oxidative stress of human erythrocytes was measured *in vitro*. Experiments were performed with a rose hip metaphosphoric acid preextract and three extracts of different polarity obtained through sequential elution of the pre-extract on a C<sub>18</sub> solid phase column.

The content of ascorbic acid in the extracts and in the different dilutions before and after enzymatic treatment is presented in Table 1. From these data, it is clear that treatment with ascorbate oxidase efficiently reduced the content of ascorbate in all samples, although minute quantities remained in extract E1B.

The Folin-Ciocalteu assay was used for measurement of the content of total phenols. A high content of phenols was found in the metaphosphoric acid extracts (PE and E1A). Although this value decreased after enzyme treatment (E1B), it was still higher than in extracts two (E2A) and three (E3A) (Table 2).

The extracts were also analysed for content of single phenolic compounds using mass spectrometry. We found significant amounts of catechin, a proanthocyanidin monomer, a proanthocyanidin dimer, rutin, quercetin galactoside, quercetin glucoside, and cyanidin-glucoside (Table 3). The metaphosphoric acid extract (E1A) contained only low levels of different phenols, whereas the second (20% methanol) extract (E2A) in particular, but also the third (100% methanol) extract (E3A) contained considerably more phenols. Of the quantified single phenolic compounds catechin and proanthocyanidins were present in highest amounts.

Using the chemical FRAP assay to measure antioxidant capacity, the metaphosphoric extract (E1A) showed the highest antioxidant capacity, 464.4 ± 84.2 μmol Fe<sup>2+</sup>/g dry weight (dw). This value decreased four-fold after enzymatic treatment to 98.0 ± 10.6 μmol Fe<sup>2+</sup>/g dw (E1B) and was then lower than in the second (20% methanol) extract (E2A) (Table 4).

This is in contrast to the biological model using erythrocytes. In Figure 1, the protection against oxidative stress on erythrocytes is shown for all extracts, both with ascorbic acid (a) and without ascorbic acid (b). Interestingly, there was a significant ( $P = 0.023$ ) increase in protection after removal of ascorbic acid, with a protection of 59.4% and 67.9% for E1A and E1B, respectively, at an extract concentration of

TABLE 2: Content of total phenols in different extracts (E1–E3, without (A) or with (B) ascorbate oxidase treatment) obtained from sequential elution of a metaphosphoric acid pre-extract (PE) of rose hips, measured by the Folin-Ciocalteu method. The bias from the ascorbic acid content in the extracts to the phenol estimates is obvious. Data are presented as mg GAE/g dw rose hip powder and mg GAE/mL extract (mean ± standard deviation).

Extract	Total phenols (mg GAE/mL)		Total phenols (mg GAE/g dw)	
	A	B	A	B
PE (HPO <sub>3</sub> )	3.0 ± 0.3		86.4 ± 8.9	
E1 (Eluent of PE)	3.4 ± 0.1	2.1 ± 0.2	24.2 ± 1.4	15.0 ± 1.3
E2 (20% MeOH)	5.2 ± 0.8	4.2 ± 0.9	12.4 ± 1.8	10.0 ± 2.0
E3 (100% MeOH)	2.0 ± 0.1	1.8 ± 0.4	4.8 ± 0.9	4.4 ± 0.8

3 mg rose hip powder per mL. E2A with the highest contents of polyphenols was the most protective of the polyphenol rich extracts with 20.8% inhibition of oxidative damage. The corresponding protection from the third extract (E3A) was 5.0%.

To confirm uptake of antioxidants by the erythrocytes, the content of polyphenols was measured in erythrocytes treated with the polyphenol rich (20% methanol) extract (E2A) using HPLC-MS. Of the studied polyphenols only catechin and proanthocyanidins entered the erythrocytes in detectable amounts (Figure 2). The uptake in the erythrocytes was very low, 3.1% of the total amount of catechin supplied (Table 5). The supernatant contained 50.7%, thus 46.2% of the added catechin remained in the pellet consisting of the erythrocyte membranes.

### 4. Discussion

Human erythrocytes are carriers of oxygen and may be exposed to reactive oxygen species which could lead to oxidative damage. Several micronutrients may protect erythrocytes against oxidative stress. In this study, we used human erythrocytes to investigate cellular protection and uptake of bioactive compounds in sequential extracts eluted from an acid water extract of rose hips. The fact that all extracts studied contributed to protective effects on the erythrocytes indicate that rose hips contain a variety of effective antioxidant compounds.

TABLE 3: Content of major single phenols in different extracts (E1A–E3A) obtained from sequential elution of a metaphosphoric acid pre-extract of rose hips. Data are presented as  $\mu\text{g}/\text{mL}$  extract (mean  $\pm$  standard deviation).

Polyphenol ( $\mu\text{g}/\text{mL}$ )	PE ( $\text{HPO}_3$ )	E1A (Eluent of PE)	E2A (20% MeOH)	E3A (100% MeOH)
Catechin	436.1 $\pm$ 77.7	7.6 $\pm$ 1.2	335.4 $\pm$ 22.8	33.8 $\pm$ 10.7
Proanthocyanidin monomer	774.4 $\pm$ 87.2	5.6 $\pm$ 2.4	625.1 $\pm$ 38.5	40.8 $\pm$ 10.4
Proanthocyanidin dimer	379.0 $\pm$ 53.8	0.0 $\pm$ 0.0	296.4 $\pm$ 26.8	10.9 $\pm$ 2.2
Rutin	44.6 $\pm$ 11.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	35.9 $\pm$ 1.1
Q-galactoside + Q-glucoside	158.5 $\pm$ 29.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	116.3 $\pm$ 1.9
Cyanidin-glucoside	44.1 $\pm$ 5.1	0.0 $\pm$ 0.0	32.0 $\pm$ 6.4	23.9 $\pm$ 3.7

TABLE 4: Antioxidant capacity of different extracts (E1–E3, without (A) or with (B) ascorbate oxidase treatment) obtained from elution of a metaphosphoric pre-extract (PE) of rose hips, measured by ferric reducing ability (FRAP). Data are presented as  $\text{mmol Fe}^{2+}/\text{L}$  extract and  $\mu\text{mol Fe}^{2+}/\text{g dw}$  rose hip powder (mean  $\pm$  standard deviation).

Fraction	FRAP ( $\text{mmol Fe}^{2+}/\text{L}$ )		FRAP ( $\mu\text{mol Fe}^{2+}/\text{g dw}$ )	
	A	B	A	B
PE ( $\text{HPO}_3$ )	58.51 $\pm$ 2.47		1712.18 $\pm$ 80.60	
E1 (Eluent of PE)	65.97 $\pm$ 11.97	13.91 $\pm$ 1.50	464.40 $\pm$ 84.25	97.95 $\pm$ 10.56
E2 (20% MeOH)	74.91 $\pm$ 12.20	59.25 $\pm$ 12.24	179.78 $\pm$ 29.28	142.20 $\pm$ 29.37
E3 (100% MeOH)	13.12 $\pm$ 2.30	9.36 $\pm$ 3.12	31.49 $\pm$ 5.51	22.47 $\pm$ 7.49

The content of ascorbic acid was highest in the pre-extract and the eluted metaphosphoric acid extract (PE and E1A). In this study, the metaphosphoric acid aqueous extracts showed superior protection against oxidative damage. In a previous study [40], we showed that the protective effect ( $\sim 75\%$ ) of rose hips could not be due to the ascorbic acid content alone as the rose hips extract only contained 0.4 mg ascorbic acid/mL in 5.0 mg dry weight rose hip powder/mL compared with a control that contained 5.0 mg ascorbic acid/mL and offered  $\sim 65\%$  protection. This implies protective capacity of notably the ascorbic acid but also of other novel compounds.

The Folin-Ciocalteu assay for measurement of total phenols has previously been found to be biased due to the interference of nonphenolic reducing compounds such as ascorbic acid and sugars [41, 42] which results in an overestimation. The contribution of ascorbic acid to total antioxidant activity can be determined, since one mole of ascorbic acid corresponds approximately to two moles of FRAP (in our assay we obtained and used the value 2.02). The contribution to the FRAP value of the ascorbic acid content of the rose hip metaphosphoric acid pre-extract was thus calculated to be 31  $\text{mmol Fe}^{2+}/\text{L}$  (Table 1), which should be compared with the obtained total FRAP value of the same extract which was 59  $\text{mmol Fe}^{2+}/\text{L}$  (Table 4). In extract E1A, the FRAP value of the ascorbic acid content was similar to the total FRAP value, indicating the contribution of ascorbic acid, but in extract E2A, the FRAP contribution of the ascorbic acid was only 6  $\text{mmol Fe}^{2+}/\text{L}$  compared with the total FRAP value of 75  $\text{mmol Fe}^{2+}/\text{L}$ . The remaining extracts made almost no contribution of FRAP ascorbic acid; hence, there were significant amounts of other compounds that contributed to the antioxidant capacity of these extracts.

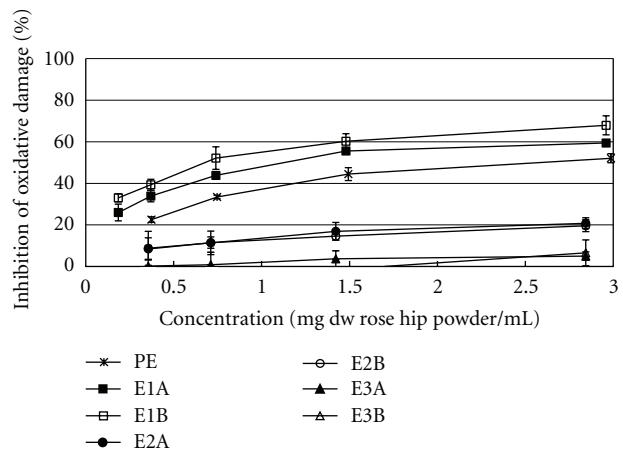


FIGURE 1: The protective effect against oxidative stress of different rose hip extracts (PE = pre-extract, E1 =  $\text{HPO}_3$  eluent, E2 = 20% MeOH eluent, E3 = 100% MeOH eluent; without (A) or with (B) ascorbate oxidase treatment) as measured on erythrocytes *in vitro*. Vertical bars represent standard deviation.

In the FRAP assay, the metaphosphoric acid extract (E1A) showed the highest ferric reducing ability, but this value decreased four-fold after enzyme treatment. The activity of the enzyme-treated extract (E1B) was lower than the secondly eluted (20% methanol) extract (E2A).

Pandey and Rizvi [13] investigated the protective effect of resveratrol on markers of oxidative stress in human erythrocytes. Human erythrocytes are able to take up resveratrol and quercetin, and once inside the cell, these compounds can donate electrons to extracellular electron acceptors through the erythrocyte plasma membrane redox

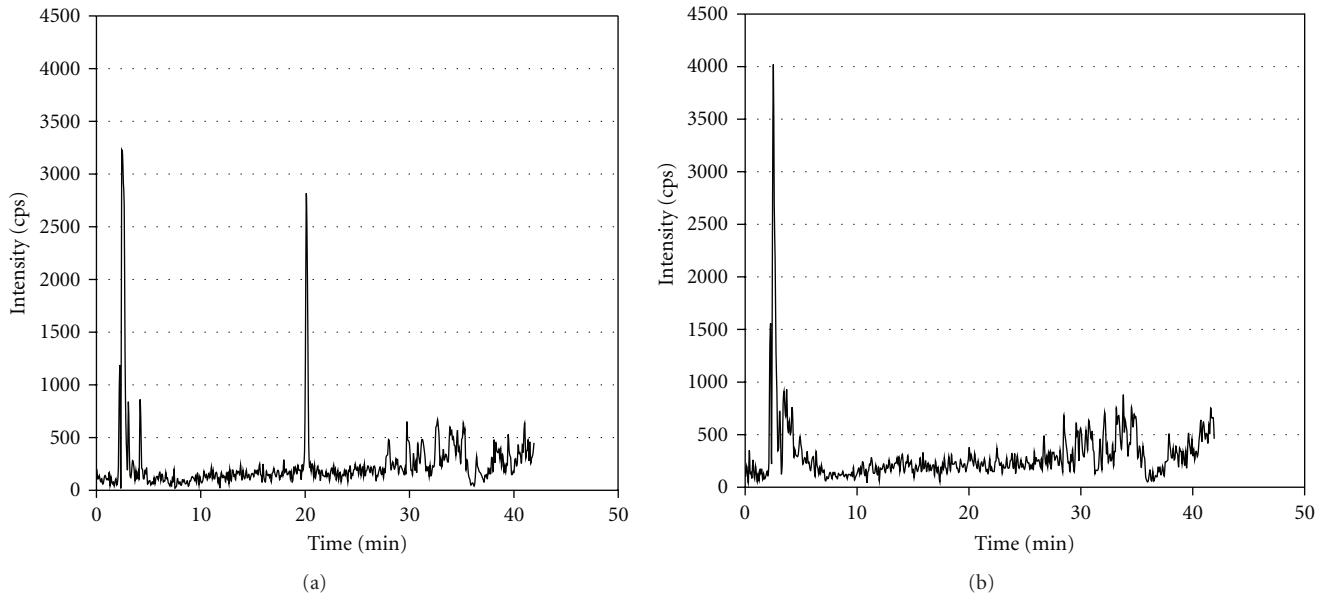


FIGURE 2: Chromatograms of erythrocytes incubated with a polyphenol rich extract (E2A) eluted from a rose hip extract (a) showing presence of catechin and control sample consisting of pure erythrocytes (b).

system. Fiorani and Accorsi [11] showed uptake of a variety of flavonoids such as quercetin, luteolin, kaempferol, fisetin, isorhamnetin, acacetin, chrysin, apigenin, galangin, and tamarixetin. Most of these agents accumulate within the cells because of their ability to bind to hemoglobin. In another study, Fiorani et al. [43] investigated flavonoids in aqueous and ether extracts and showed that polyphenols in ether extracts elicited their antioxidant effects by activation of plasma membrane oxidoreductase but that flavonoids in aqueous extracts were ineffective in the cell-based assay. The authors accounted for these observations through the inability of aqueous components to cross the erythrocyte membrane [43]. In our study, the protective capacity was most obvious in the aqueous extracts. The highest degree of protection was obtained with the first eluted metaphosphoric acid extract (E1A), and, interestingly, there was an increase in protection of erythrocytes after removal of ascorbic acid from this eluent (E1B). Ascorbate oxidase efficiently reduced the content of ascorbate in this extract, E1B, with only minute quantities remaining. However, extract E1B most likely contained DHA, which has equivalent biological activity to ascorbic acid as erythrocytes have a high capacity to regenerate ascorbic acid. Erythrocytes lack an active transporter for ascorbate, whereas DHA is rapidly taken up by facilitated diffusion by a glucose transport protein, GLUT1. Intracellular DHA is rapidly reduced to ascorbate by GSH in a direct chemical reaction and trapped within the cell [44]. Enzyme-dependent mechanisms involving both glutaredoxin and thioredoxin reductase have also been demonstrated [44]. The increased protection obtained with the enzyme-treated extract (E1B) may thus be explained by reactivated ascorbate and GSH. Haemoglobin is a reactive protein, and erythrocytes stabilize and protect it with

intracellular GSH levels. The GSH maintenance system provides the reducing power to maintain other cellular antioxidants in their reduced states. The uptake of DHA into the cells may have temporarily depleted intracellular thiol levels, although any significant reduction in GSH levels and subsequent rise in GSSG will immediately stimulate NADPH formation by the hexose monophosphate shunt (HMP) [9]. The HMP activity will then restore GSH levels, and the cells will have reactivated ascorbate and GSH, which might account for the increased protection of the enzyme-treated extract. It may also be that the major contribution to total antioxidant activity could have come from a combination of phytochemicals, not from ascorbic acid alone, as previously shown by Sun et al. [45].

To confirm antioxidant uptake, the content of catechin was measured by HPLC-MS. The uptake was only 3.1% of the total amount of catechin supplied. The supernatant contained 50.7%, thus 46.2% of the added catechin remained in the pellet consisting of the erythrocyte membranes. This is in agreement with Koren et al. [34], who showed that human erythrocytes not only carry oxygen but also have the ability to bind polyphenol antioxidants.

## 5. Conclusions

The fact that all sequentially eluted extracts studied contributed to protective effects on the erythrocytes indicate that rose hips contain many different antioxidant compounds. The aqueous metaphosphoric acid extracts showed the highest protection against oxidative damage, this implies protective capacity of the ascorbic acid as well as other unidentified compounds.

TABLE 5: Mean content of major polyphenols  $\mu\text{g/mL}$  (mean  $\pm$  standard deviation) in erythrocytes incubated with the polyphenol rich rose hip extract E2A (eluted with 20% methanol).

Polyphenol	Polyphenols ( $\mu\text{g/mL}$ )
Catechin	$0.321 \pm 0.011$
Proanthocyanidin monomer	$0.002 \pm 0.003$
Proanthocyanidin dimer	$0.125 \pm 0.190$

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