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ORIGINAL CONTRIBUTION

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Rosa roxburghii supplementation in a controlled feeding study increases plasma antioxidant capacity and glutathione redox state

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Summary *Background Rosa roxburghii* (*RR*) is a plant of which the fruit juice has been used as a medicinal remedy for a variety of diseases. It has been proposed that the putative beneficial properties are related to its antioxidant potential. *Aim of study* We investigated the contribution of a supplemented *RR* fruit sample on the antioxidant status in a cohort of healthy humans. *Methods* A total of 36 young, healthy and non-smoking individuals were recruited for this randomised placebo-controlled, single-blind trial. The study was diet controlled over a five-week period with a two week run-in period before participants daily received a placebo or an encapsulated supplement of *RR* sample. Total antioxidant capacity, glutathione redox state, glutathione reductase, glutathione peroxidase, superoxide dismutase and 8-OHdG levels were measured. *Results RR* supplementation significantly increased plasma antioxidant capacity ($p = 0.04$) and GSH:GSSG ratios in blood ($p = 0.03$). No significant changes in 8-OHdG levels, total glutathione levels or antioxidant modulating enzymes were detected suggesting that the observed shift of the glutathione redox state probably occurs via the antioxidant mediated protection of GSH. *Conclusions* We conclude that these findings support the putative beneficial properties that have been linked to *Rosa roxburghii* as a dietary supplement that can enhance antioxidant status.

Key words Rosa roxburghii – antioxidants – glutathione redox state – polyphenols – antioxidant capacity

Introduction

Rosa roxburghii (*RR*), also known as sweet chestnut rose, is a wild plant of the rose family that originates from China. Fruit extracts of this plant have been used for medicinal purposes and it is believed to have a protective effect against various diseases, including arteriosclerosis, cancer, and immunity stress [1]. The putative beneficial effects of this plant extract have been associated with antioxidant components resulting in an increase in the antioxidant status in erythrocytes of healthy aged participants [2]. An extract of the fruit contains, amongst others, notable amounts of ascorbic acid and as we report here polyphenols and to a lesser extent vitamins E, B1, zinc and calcium [1]. It has also been suggested that a high superoxide dismutase (SOD) content contributes significantly towards its beneficial properties [3, 4], although the mechanism for this is not clear.

The putative beneficial effect of *RR* extract for the prevention or treatment of various diseases could be a result of a combination of the various compounds that exist in this fruit. Antioxidants act synergistically and it is suggested that combinations of antioxidants may be more effective than larger quantities of a single antioxidant [5]. A previous investigation using aged individu-

EJN 555

als indicated that dietary supplementation with *RR* increased the activities of SOD and catalase and reduced glutathione in blood, although it was not clear if the study included a controlled feeding protocol [2]. Since antioxidants are commonly present in the diet, it is essential to support the putative antioxidant potential of *RR* in a controlled feeding study.

We investigated the effect of dietary supplementation with *RR* extract on selected markers for antioxidant status in healthy individuals in a randomised placebo-controlled, controlled-feeding study. We also provide a breakdown of the major known antioxidant compounds in the *RR* sample used. Although it is expected that oxidative stress-related parameters do not change significantly in healthy young individuals who follow a healthy diet, it has been shown that an increase of antioxidant status can be expected with a higher consumption of fruit and vegetables [6, 7]. For this study we therefore only included healthy individuals to serve as a good test for investigating the effect of *RR* on antioxidant status.

Methods and materials

Rosa roxburghii fruit antioxidants analyses

A concentrated sample of the juice of the *RR* juice (*RR* sample) was supplied by G. Joubert. This sample was analysed for ascorbic acid and polyphenol content as previously described [8, 9]. The antioxidant capacity of the sample was determined using the oxygen radical absorbance capacity (ORAC) method [10]. Polyphenol components in the *RR* sample were extracted and measured in a diluted *RR* sample by GC-MS essentially as described elsewhere [11].

Study design and subjects

The study was a randomised, paired, placebo-controlled, single-blind parallel study. Informed written consent from participants and ethical approval from the Ethics Committee of the North-West University (project 02M06) were obtained. Thirty-six healthy volunteers of equal gender, aged between 21–27 years were recruited following completion of a questionnaire that included medical and diet-related questions. Exclusion criteria included a history of cardiovascular, hepatic, gastrointestinal or renal disease, frequent alcohol use, use of antibiotics or any dietary supplements within four weeks prior to the start of the study, smokers and excessively physically active persons. Women who were pregnant or lactating or using birth control pills or who had a history of menstrual irregularities were also excluded.

All participants lived at home but took all meals at a central location throughout the five-week study period. The meals consisted of a typical diet for the participants and the composition was recorded to calculate the nutritional value (Table 1). For the first two weeks participants followed the prescribed diet without supplements. Based on age, gender, and body mass index (BMI), subjects were then paired and randomly allocated to either an intervention or a placebo group. The mean BMI was 22.7 ± 2.7 kg/m^2 and 22.8 ± 3.0 kg/m^2 for the placebo and intervention groups, respectively. The intervention period was 21 days during which each participant received four capsules to be taken with every meal. The capsules contained either a concentrated *RR* sample that was concentrated by low-temperature vacuum, or a talk placebo. The four *RR* capsules represented an original equivalent of 24 ml of the *RR* fruit sample, which is similar to what is commonly/traditionally consumed with this fruit juice. In total, each person in the intervention group daily received from the supplement a total of 1.55 g polyphenols and 1.08 g ascorbic acid which contributed to a total antioxidant capacity of 28 mmoles trolox equivalents.

The use of antioxidant supplements or alcohol was prohibited during the study period. From the average intake over a three day diet period, the percentages of energy from protein, fat, and carbohydrate were 15 %, 39 %, and 45 %, respectively (Table 1). Participants maintained

Table 1 Daily consumption of macronutrients and antioxidants

Macro/micronutrients	Mean ± SD	Max	Min	DRI* male (19–30 y)	DRI* female (19–30 y)
Total energy (kJ)	11546 ± 1446	13109	8168	12881	10093
Proteins (g)	104 ± 21	132	73.6	56	46
Fats (g)	119 ± 23	152	72.8	–	–
Carbohydrates (g)	307 ± 47	410	243	130	130
Vitamin A (RE)	3487 ± 2367	6726	610	900	700
Vitamin C (mg)	141 ± 114	347	41	90	75
Vitamin E (TE)	14 ± 5	23	4.3	15	15

* *DRI* dietary reference intakes [16]. The values do not include the contribution of the *Rosa roxburghii* supplement

their usual daily activities and recorded any deviations from the proposed diet including medication and unusual physical activity. Dietary intakes were calculated using the nutrient content according to the menus served to the participants by using the Food Finder (MRC, MedTech, Cape Town, South Africa) dietary analysis software. Compliance with the diet was monitored by keeping records of meals, medicine taken and any possible intake of food or beverages between meals.

Blood and urine samples

Fasting (12 hour) blood and urine samples were taken before and after the 21-day intervention period. Venous blood samples were collected without stasis between 07:00 and 10:00 to avoid the effect of diurnal variation. Urine samples were stored at –20 °C until analysis.

Antioxidant capacity

Plasma antioxidant capacity of plasma was determined by the ORAC method essentially as described before [10]. Deproteinated (perchloric acid treated) samples were prepared immediately and frozen at –80 °C until use. AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) was used as a peroxyl radical generator and trolox was used as a standard. The total plasma antioxidant capacity was expressed as μM trolox equivalents (TE). Total plasma polyphenol content was determined as described before [9], with quercetin used as a standard in the assay.

Blood glutathione and enzyme analyses

The redox state of glutathione was measured in EDTA blood using the spectrophotometric GSH/GSSG ratio assay kit (GSH/GSSG-412™) from OXIS Research. Blood samples were immediately prepared for separate total GSH and GSSG measurements as suggested by the supplier. Erythrocyte SOD activity was determined by monitoring the auto-oxidation of pyrogallol [12], and glutathione reductase (GR) and glutathione peroxidase (GSH-PX) were measured in erythrocytes as described previously [13, 14]. Enzyme activities were performed within hours after blood was drawn and expressed as units per milligram of haemoglobin, which was measured using the Hb assay reagents from Roche.

Full blood counts

Full blood counts in freshly obtained EDTA-blood were measured using a Coulter counter (Beckman, A^{c}T™ 5diff Cap Pierce Haematology Analyzer) according to the manufacturer's instructions. No significant changes in any of the blood cell parameters occurred at any time during the study (results not shown).

Urinary 8-hydroxy-deoxyguanosine (8-OH-dG)

An estimation of DNA oxidation was performed by measuring 8-OH-dG levels in urine samples using an immunological detection assay (8-OHdG-EIA™, OXIS Research), and creatinine values were measured in the urine samples based on the commonly used Jaffé reaction.

Statistical analysis

All analyses, except GC-MS analysis, were performed in duplicate and the average used in subsequent data analyses. Data were analysed with *Statistica Version 6* software. Normal distribution of data was tested via Shapiro Wilk's W test. The Wilcoxon matched pair test (non-parametric) was used to test for significant changes due to the *RR* supplementation. The Mann-Whitney test was used to test for parameter changes between groups, and a p-value below 0.05 was considered to be significant. Values reported in the text are mean ± standard deviation.

Results

Antioxidants in the *RR* sample

The antioxidant capacity of the sample was determined to be 1154 mM TE. The total ascorbic acid content was 45.4 g/l, of which 20 % was in the reduced form and the total polyphenol content was 64.9 g/l gallic acid equivalents (GAE). A summary of the polyphenol compounds in the *RR* sample is given in Table 2. Using the published trolox equivalent antioxidant capacity (TEAC) of the various compounds, the contribution of individual polyphenols to the total antioxidant activity (TAA) of the sample was calculated [15]. Considering these values, quercetin, epicatechin and catechin contribute significantly to the TAA, although the largest contribution comes from the non-flavonoid components, including pyrogallol, gallic acid, m- and p-coumaric acid.

Diet and compliance

The macronutrient and average antioxidant intake during the study period are summarised in Table 1. Although the compliance of the participants in the study

Table 2 Polyphenol content of *Rosa roxburghii* juice

Class	Compound	Conc. (g/L)	TEAC (mM)	TAA (mM)
Non-flavonoids				
• Hydroxybenzenes	Pyrocatechol	0.47	1.45	6.2
	Pyrogallol	1.23	1.91	18.67
• Hydroxybenzoic acids	Salicylic acid	0.13	0.04	0.04
	m-Hydroxybenzoic acid	0.50	0.84	3.10
	Vanillic acid	0.33	1.43	2.82
	Gentisic acid	0.45	1.04	3.05
	Homogentisic acid	0.05	0.91	0.32
	Gallic acid	3.62	3.01	64.16
	α-Resorcylic acid	0.14	2.15	1.92
	Protocatechuic acid	0.93	1.19	7.15
	Syringic	0.05	1.36	0.37
• Hydroxycinnamic acids	m-Coumaric acid	1.51	1.21	11.17
	p-Coumaric acid	1.35	2.22	18.23
	Ferulic acid	0.21	1.91	2.10
	Isoferulic acid	0.13	*	*
	Caffeic acid	0.21	1.26	1.49
• Hydroxyhydrocinnamic acids	Hydrocaffeic acid	0.30	1.43	1.67
	Hydro-p-coumaric acid	0.46	*	*
	Hydroferulic acid	0.24	*	*
• Hydroxyphenylacetic acids	p-Hydroxyphenylacetic acid	0.10	0.64	0.34
	3-Methoxy-4-hydroxyphenylacetic acid	1.72	0.23	6.02
Flavonoids				
• Flavon-3-ols	Quercetin	0.31	4.72	4.93
	Morin	0.32	2.55	2.67
• Flavonones	Taxifolin	0.28	1.9	1.78
• Catechins	Catechin	1.32	2.4	10.92
	Epicatechin	1.38	2.5	11.87
	Epigallocatechin	0.07	3.82	0.93

TEAC trolox equivalent antioxidant capacity; *TAA* the contribution of the compounds to the total antioxidant activity of the mixture [15]; * Data unavailable

was not perfect, the two groups compared well with regard to adhering to the prescribed diet. Three participants, two in the placebo and one in the intervention group, were excluded due to insufficient compliance with the protocol.

Plasma antioxidant capacity

The mean baseline fasting deproteinated plasma antioxidant capacity values were 1164 ± 316 μM TE for the placebo group and 1174 ± 249 μM TE for the intervention group (Fig. 1A). These values are similar to reported values for healthy individuals using the ORAC method (1200–1300 μM TE) [17]. The antioxidant capacity after the intervention period was 1146 ± 162 μM TE for the placebo group which is not significantly different from baseline values. In the intervention group, however, plasma ORAC values increased significantly to 1309 ± 272 μM TE ($p = 0.04$) (Fig. 1A). The net increase was also significant when comparing the net change over the intervention period in the placebo group with the intervention group ($p = 0.02$). Total plasma polyphenols did not change significantly over the intervention period with values ranging from 272 ± 53 to 289 ± 17 mg/l for the placebo group over the intervention period and 277 ± 46 to 287 ± 34 mg/l for the intervention group.

Glutathione redox state

Reduced glutathione (GSH) levels in the placebo group remained relatively constant over the intervention period with levels of 1109 ± 235 μM before and 1063 ± 238 μM after intervention. In the intervention group a slight, although insignificant increase from 1021 ± 219 μM to 1071 ± 216 μM occurred. However, a decrease from 2.3 ± 0.9 μM to 1.6 ± 0.7 μM was observed in the GSSG levels of the intervention group. The resulting glutathione redox state (GSH/GSSG, Fig. 1B) thus changed significantly from 521 ± 225 to 696 ± 263

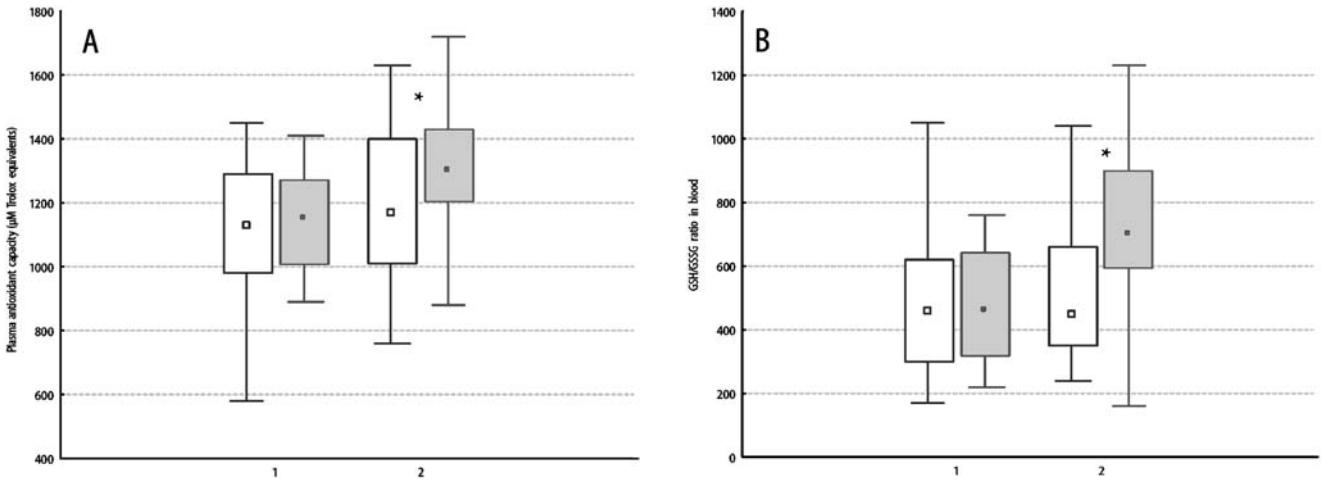

Fig. 1 Plasma antioxidant capacity and glutathione redox state of study participants. Plasma antioxidant capacity (**A**) and whole blood glutathione redox state (**B**) in participants receiving placebo [1] or *Rosa roxburghii* sample [2] are shown. The open boxes indicate the levels at the start of the intervention period and the grey boxes indicate the levels after the 21 day intervention period. Boxes indicate 25–75 percentiles and the marks inside the boxes indicate median values. Whiskers indicate minimum and maximum outliers. The asterisks in **A** and **B** indicate where statistical significant differences were observed (p = 0.004) using the Wilcoxon matched pairs test. Each individual sample data point used in the analysis was the average of a duplicate assay

($p = 0.03$) in this group. In the placebo group no significant changes were observed, i. e. the GSSG levels remained relatively unchanged at 2.7 ± 1.1 µM before and 2.4 ± 0.9 µM after intervention with the GSH/GSSG ratio ranging from 484 ± 240 before the intervention period to 492 ± 185 afterwards.

Erythrocyte SOD, GR and GSH-PX activities

The activities of the three enzymes measured in erythrocytes remained relatively constant in both groups over the intervention period (Table 3). No significant changes were observed in any of these parameters.

Table 3 Erythrocyte superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase activities in study participants

	Placebo group*		Intervention group*	
	Baseline	Day 21	Baseline	Day 21
SOD (U/mg Hb)	1.16±0.1	1.18±0.1	1.19±0.1	1.18±0.1
GR (U/g Hb)	8.33±1.1	8.23±1.2	8.38±0.9	8.21±0.8
GSH-PX (U/g Hb)	22.1±3.5	23.0±2.6	21.3±3.4	21.7±3.5

* The placebo group included 16 individuals and the intervention group 17. All assays where performed in duplicate and with the median of the group ± SD indicated

Urinary 8-OH-dG

The urinary 8-OH-dG levels, which were normalized against creatinine levels, also showed no significant changes. The levels in the placebo group changed slightly from 15.7 ± 4.8 to 14.0 ± 4.4 ng/mg creatinine and the intervention group remained relatively constant at 19.1 ± 6.5 and 18.9 ± 5.0 ng/mg creatinine over the intervention period.

Discussion

The fruit of the *Rosa roxburghii* plant is somewhat of an enigma in western countries which is, in part, due to its origin and limited availability as well as its remarkably high antioxidant content. *In vitro* studies suggest that the fruit juice is not mutagenic, cytotoxic or genotoxic and protects against *tert*-butyl hydroperoxide-induced oxidative stress (unpublished data). Although some data exist on its putative beneficial properties as a dietary supplement in aged individuals, there are still limited data available on its properties and ability to contribute to antioxidant status.

It was demonstrated here in healthy individuals that selected markers of antioxidant status, i. e. antioxidant capacity and glutathione redox state improved significantly after only three weeks of supplementation with *RR*. Significant changes in plasma total polyphenol levels could not be demonstrated, although the bioavailability and half life of these compounds varies greatly and their contribution to antioxidant status may remain undetectable before a prolonged supplementation

would allow significant levels to become detectable [18]. The relatively high dietary ascorbic acid intake of ± 140 mg/day in this study would probably not allow additional increases in ascorbic acid (not measured), as it was already higher than the sigmoidal dose responsiveness of ascorbic acid intake [19]. We believe, however, that changes (albeit undetectable) in both polyphenols and ascorbic acid levels, in addition to other less abundant compounds that exist in the juice such as zinc and α-tocopherol, nevertheless could collectively contribute to the observed general shift in antioxidant capacity, protecting free reduced glutathione and thus shifting the available glutathione pool towards a reduced state. Our data strongly suggest that the observed shift in glutathione redox state was not due to synthesis or via enzymatic/allosteric modulation [20]. The *RR* juice is a relatively poor source of L-cystein (1.4 mg/l), which is a substrate for GSH synthesis, and therefore total glutathione remained relatively constant. Both glutathione reductase and glutathione peroxidase remained unchanged in the participants and, in addition, polyphenols that could allosterically modulate glutathione redox state [21] were not detected in the juice.

The increase in antioxidant status with *RR* supplementation, however, were not reflected in levels of urinary excretion of 8-OHdG, which may be due to the fact that healthy young individuals were used in this study where oxidative stress should be much less as compared to aged or diseased individuals.

Although this study was limited in terms of its scale and scope of parameters investigated, we conclude that our results support the potential of *Rosa roxburghii* as a supplement that promotes antioxidant status. Further studies are now warranted to investigate its potential as a useful natural supplement in maintaining health and preventing disease states and particularly those characterized by disturbances in oxidative status.

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