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Communication

Carotenoid composition and *in vitro* pharmacological activity of rose hips*

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The aim of the present study was to compare carotenoid extracts of Rose hips (*Rosa canina* L.) with regard to their phytochemical profiles and their *in vitro* anti-*Helicobacter pylori* (*H. pylori*), cytotoxic, multidrug resistance (MDR) reversal and radical scavenging activity. Carotenoid composition was investigated in the different fractionation of rose hips, using extraction methods. Six main carotenoids — epimers of neochrome, lutein, zeaxanthin, rubixanthin, lycopene, β,β-carotene — were identified from Rose hips by their chromatographic behavior and UV-visible spectra, which is in accordance with other studies on carotenoids in this plant material. The active principles in the carotenoid extract might differ, depending upon the extraction procedures.

Key words: *Rosa canina* L., carotenoids, cytotoxic activity, anti-*H. pylori*, MDR, radical intensity

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INTRODUCTION

Carotenoids play an important role in preventing oxidative damage which are caused by free radicals in age-related diseases such as cancer and ageing (Britton *et al.*, 2005; Motohashi *et al.*, 2003; Krinsky & Johnson, 2005). Most current research is focused on a proposed role of carotenoids as lipid antioxidants which can protect the cells or hosts from the oxidation and other destructive processes mediated by singlet oxygen and free radicals, though more specific effects on the immune system are now under investigation (Britton *et al.*, 2005; Krinsky & Johnson, 2005). In this study, we present qualitative and quantitative data on the carotenoid content of Rose hips. We investigated anti-*Helicobacter pylori* (*H. pylori*), cytotoxic, multidrug resistance (MDR) reversal and radical scavenging activity by the carotenoid extracts of Rose hips.

MATERIALS AND METHODS

Chemicals and reagents. They were obtained from the indicated companies: RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), metronidazole, ascorbic acid, Rose Bengal and 2,2,6,6-tetramethyl-4-hydroxy-piperideine (4-OH TEMP) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chem. Co., St. Louis, MO, USA); dextran sulphate (DS, 8 kD), (Kowa Chem. Co., Tokyo, Japan); β,β-carotene and gallic acid (Tokyo Kasei Co., Tokyo, Japan); clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan). α-Tocopherol was a gift from Eisai Ltd. (Tokyo, Japan). A strain of *H. pylori* (ATCC 43504) was purchased from American Type Culture Collection (Rockville, MD, USA).

Isolation of phytoxanthins from Rose hips (*Rosa canina* L.). Rose hips (1.2 kg fresh weight) have been collected in the surroundings of Pécs (Hungary). Isolation of PM2 and PM9-PM12 in crystalline state was carried out in our laboratory according to reference of Molnár *et al.* (2005a). PM2: Phytoxanthins of Rose hips, 680 mg crystalline sample; PM9: Hypophasic carotenoids of the ethereal extract of Rose hips, 176 mg crystalline sample; PM10: Epiphasic carotenoids of the ethereal extract of Rose hips, 350 mg crystalline sample; PM11: Hypophasic carotenoids of the MeOH extract of Rose hips, 96 mg crystalline sample; PM12: Epiphasic carotenoids of the MeOH extract of Rose hips, 180 mg crystalline sample.

HPLC analysis of samples PM2, PM9-12. HPLC was performed on a Dionex 580P NDG pump (Dionex Softron Germering, Germany) connected to a Dionex PDA-100 detector running Dionex Chromeleon software. Column: 250 mm × 4.6 mm i.d., 6 mm, endcapped Chromsyl C18. Gradient elution was performed with mixture of mobile phases as follows: A, 12% H_2O in MeOH; B, MeOH: ad C, 30% CH_2Cl_2 in MeOH. Gradient program: 0–2 min, 100% A; 2–10 min, to 80% A 20% B; 10–18 min, to 50% A 50% B; 18–25 min, to 100% B; 25–27 min, 100% B, 27–34 min, to 100% C; 34–41 min, 10% C (in linear steps); flow rate: 1.2 ml/min. Peak identification was at 450 nm (Deli *et al.*, 2000). Carotenoids were identified on the basis of their UV-Vis spectroscopic

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Abbreviations: 4-OH TEMP, 2,2,6,6-tetramethyl-4-hydroxy-piperideine; 4-OH TEMPO, 2,2,6,6-tetramethyl-4-hydroxy-1-piperidinyloxy; BHI, brain heart infusion; DMEM, Dulbecco's modified Eagle medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FBS, fetal bovine serum; HGF, human gingival fibroblasts; HSC-2, human oral squamous cell carcinoma; HSG, human submandibular gland tumor; MDR, multidrug resistance; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

properties in different solvents, chemical reactions [(*E*/*Z*)-isomerization (thermal isomerization and iodine-catalized photoisomerization), 5,6-epoxide → 5,8-epoxide (furanoid oxide) rearrangement, $NaBH_4$-reduction], by co-chromatography with authentic reference samples (purity: >95%; HPLC) and by HPLC retention times (Deli *et al*., 2000).

Measurement of anti-*H. pylori* activity. The microdilution broth method was used to determine the minimum inhibitory concentration (MIC). Brain Heart Infusion (BHI) broth containing 10% fetal bovibe serum (FBS) and 0.1% glucose was used as the medium and was cultured in a jar conditioned with AnaeroPack (Campylo, Mitsubishi Gas Chemical Co., Inc.). *H. pylori* was inoculated in the medium and cultured at 37°C for 2 days. The collected bacterial colonies were diluted to about 10^7 colony forming unit (CFU)/ml with the medium. The fractions were dissolved in DMSO, and then diluted with the medium. To the solution of the fractions, a suspension of bacteria was added to make 10^6 CFU/150 mL/well. The mixture was incubated at 37°C for 5 days. The MIC values of the fractions were determined by observation (Kawase *et al*., 2003).

Cell culture. Human oral squamous cell carcinoma (HSC-2) cells (provided from Prof. Nagumo, Showa University) and human submandibular gland tumor (HSG) cells (provided from Dr. Atsumi, Meikai University) were maintained as monolayer cultures at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-activated FBS in a humidified 5% CO_2 atmosphere, and subcultured by trypsinization. Human gingival fibroblasts (HGF) were isolated from the periodontal tissue of healthy gingival biopsy of a 10-year-old female, as described previously (Sakagami *et al*., 2000), according to the guideline of Meikai University Ethic Committee, after obtainig the informed consent from the patients. Cells between the fifth and seventh passages were used.

Measurement of cytotoxic activity. Near confluent HSC-2 and HSG cells were incubated for 24 h with the indicated concentrations of test samples in culture medium in 96-microwell plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the viable cell number was determined by MTT method (Sakagami *et al*., 2000). The cells were washed with PBS, and incubated for 4 h with fresh culture medium containing 0.2 mg/mL MTT. After removing the medium, cells were lysed with 100 mL DMSO and the absorbance at 540 nm of the cell lysate was measured with Labsystems Mutiskan^R with Star/DOT Matrix printer JL-10. The A_{540} values of control HSC-2, HSG and HGF cells were 1.519, 0.884, and 0.283, respectively. We found that the linearity of the absorbance value was maintained between 0–2.0. The absorbance value of 1.0 was correlated to approximately 4×10^4 cells/cm^2. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Measurement of the MDR reversal activity. The *MDR1*/A expressing cells were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype (Kessel, 1989). The L5178 MDR cell and the L5178 Y parent cell were grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2×10^6/ml and resuspended in serum-free McCoy's 5A medium, and 0.5 ml aliquot of the cell suspension were distributed into each Eppendorf centrifuge tube. Then 10 µl of 2 mg/ml test compounds (dissolved in DMSO) were added and incubated for 10 min at room temperature. Then 10 µl rhodamine 123 as indicator of drug accumulation was added to the extracts (5.2 µM final concentration) and the cells were incubated for a further 20 min at 37°C, washed twice and resuspended in 0.5 ml PBS (pH 7.4) for analysis. The fluorescence intensity of cell population was measured by flow cytometry (instrument: Beckton Dickinson FACScan). (±)-Verapamil was used as the positive control in R123 accumulation experiments (Weaver *et al*., 1993). The R123 accumulation was calculated from fluorescence of one height values. Then, the percentage of mean fluorescence intensity was calculated in treated *MDR1* and parental cells, compared to untreated cells. The fluorescence activity ratio was calculated by following equation (Weaver *et al*., 1993):

MDR1 reversal activity = (*MDR1* treated/*MDR1* control)/(parental treated/parental control)

Measurement of the radical scavenging activity

DPPH scavenging assay. For the determination of DPPH radical, 30 µL of 300 µM DPPH in EtOH were added to 60 µL of sample in 40% DMSO solution and analyzed 1 min after mixing. The microwave power, gain and time constant were changed to 5, 400 and 0.1 s, respectively (Molnár *et al*., 2005b).

$^1O_2$ scavenging assay. The sample was added to the reaction mixture of 20 mM 4-OH TEMP and 20 µM Rose Bengal in 20% DMSO solution and the mixture in flat cell was irradiated for 5 min at 550 nm and analyzed by using ESR spectrometer. The microwave power, modulation width, the gain and time constant were changed to 5, 0.2, 200 and 0.1 s. Singlet oxygen was measured by oxidation of 4-OH TEMP to 2,2,6,6-tetramethyl-4-hydroxy-1-piperidinyloxy (4-OH TEMPO) radical (Molnár *et al*., 2005b).

RESULTS AND DISCUSSION

Carotenoid composition of PM2, and PM9-12 fractions

On the basis of UV-VIS spectrum of the peaks, as well as the retention time and co-cromatography with authentic reference samples, the following carotenoids were identified in PM2 fraction (Molnár *et al*., 2001): antheraxanthin: 5.9%; epimers of mutatoxanthin: 5.1%; lutein + zeaxanthin: 59.7%; (Z)-isomers of lutein and zeaxanthin: 1.5%; rubixanthin + β-cryptoxanthin: 11.7%; lycopene + (Z)-lycopene: 7.4%; β,β-carotene: 1.5%. In the fraction of PM9 the following carotenoids were identified: (all-E)-neoxanthin (1.3%); (9'Z)-neoxanthin (2.1%); epimers of neochrome (8.8%); epimers of auroxanthin (6.3%); lutein + zeaxanthin (74.7%); (Z)-isomers of lutein and zeaxanthin (2.1%); rubixanthin + β-cryptoxanthin (0.9%). The main carotenoids of PM10 fraction were lycopene (62.3%) and β,β-carotene (23.9%). The fraction of PM11 contained epimers of neochrome (6.1%); epimers

Table 1. Anti-*H. pylori* activity of Rose hips carotenoids

Extract	*H. pylory* (MIC_{50}, µg/ml)
PM2	90
PM9	21
PM10	8.2
PM11	11
PM12	40
Metronidazole	7.0
Amoxicilin	0.023

Table 2. Cyctotoxic activity of Rose hips carotenoids against tumor and normal cells

Compound	50% cytotoxic concentration (CC_{50}, μg/ml)									SI[a]= CC_{50} (normal)/ CC_{50} (tumor)
	Human tumor cell						Normal cell			
	HSC-2	HSC-3	HSC-4	HepG2	T98G	HL-60	HGF	HPC	HPLF	
PM2	73	81	50	84	>100	15	89	69	96	<1.3
PM9	>200	98	77	188	>200	15	195	154	191	>1.4
PM10	44	79	36	71	160	14	144	85	152	1.9
PM11	12	38	23	14	68	3	78	25	98	2.5
PM12	154	161	104	145	>200	39	>200	85	>200	><1.2
β,β-carotene	193	>200	–	–	-	>200	>200	>200	>200	><1.0
Gallic acid	19	29	–	–	-	24	81	75	49	2.48
A_{540}	1.3	0.6	0.81	0.34	0.49		0.56	0.5	0.51	

Near confluent cells were incubated for 24 hours without or with various concentrations of each compound, and the relative viable cell number (absorbance at 540 nm of the MTT-stained cell lysate) was determined by the MTT method. The viable cell number of HL-60 cells was determined by trypan blue exclusion. The CC_{50} was determined from the dose-response curve. Each value represents the mean from duplicate determinations. [a]determined by the equation: SI= [CC_{50}(HGF) + CC_{50}(HPC) + CC_{50}(HPLF) / CC_{50}(HSC-2) + CC_{50}(HSC-3) + CC_{50}(HSC-4)+ CC_{50}(HepG2) + CC_{50}(T98G) + CC_{50}(HL-60)] × 6/3.

of auroxanthin (13.0%); antheraxanthin: 2.3%); lutein + zeaxanthin (53.7%); (Z)-isomers of lutein and zeaxanthin (7.3%); and rubixanthin + β-cryptoxanthin (2.8%). In the PM12 fraction lycopene (27.5%) and β,β-carotene (40.4%) were identified as the main components.

Anti-*H. pylori* activity

Helicobacter pylori is an important human pathogen that infects up to 50% of the human population. As the leading cause of peptic ulcers, gastritis and gastric cancer worldwide, the organism has been the subject of intensive research to unravel the mysteries of its genetics and cellular biology. Antibacterial agents from a diet rich in fruit and vegetable may be superior as *H. pylori* control agents, when compared to many non-natural products (Kawase & Motohashi, 2004). Carotenoids, as free radicals, play an important role in the pathogenesis of gastroduodenal mucosal inflammation, peptic ulcer disease, various micronutrients are considered to protect the gastric mucosa by scavenging the free radicals. Among the tested PM2 and PM9-12 extracts only the PM10 and 11 displayed some anti-*H. pylori* effects (MIC_{50}= 8.2 μg/ml and 11 μg/ml) comparable to metronidazole (MIC_{50}= 7.0 μg/ml) (Table 1). The other compounds PM2, 9 and 12 had moderate activity.

Cytotoxic activity

Carotenoids showed potent *in vitro* anti-tumor promoting activity (Garewal, 1993). A higher intake of carotenoids-rich foods is associated with a reduced risk of

Table 3. Effect of Rose hips carotenoids on the multidrug resistance of L-5178 cells

Extract	Concentration (μg/ml)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
Par (control)[b]	–	546.05	224.80	936.73	
MDR + R123 (mean)[c]	–	604.47	303.43	11.81	–
(**dl**)-Verapamil	10	581.09	296.13	86.27	7.30
DMSO	20	573.37	306.12	14.65	1.24
PM9	4	602.90	310.22	27.78	2.35
	40	585.17	303.20	167.97	14.22
PM10	4	610.71	309.18	25.62	2.17
	40	576.39	303.77	134.76	11.41
PM12	4	634.75	332.75	16.18	1.86
	40	591.85	328.64	122.92	14.15
β,β-carotene	4	483.20	200.85	396.50	46.32
	40	493.89	197.99	1612.27	188.34

[a]FSC: Forward scatter count; SSC: Side scatter count; FL-1: Fluorescence intensity; [b]Par: a parenteral cell without MDR gene; [c]MDR: a parenteral cell transfected with MDR gene.

Table 4. Radical scavenging activity of Rose hips carotenoids

Extract	DPPH radical intensity		Singlet oxygen intensity	
	concentration (μg/ml)	%	concentration (μg/ml)	%
control	0	100[a]	0	100[b]
PM9	1000	51.5	400	97.7
PM10	1000	58.0	400	89.2
PM12	1000	36.6	400	87.6
β,β-carotene	1000	6.2	400	80.2

[a]Radical intensity of DMPO-OOH in control was 1.105. [b]Radical intensity of DPPH in control was 1.977. Each value represents mean of triplicate assays. S.D.<10%.

the development of cancer formation. Capsanthin and related carotenoids isolated from the fruits of red paprika exhibited potent antitumor-promoting activity (Maoka *et al*., 2001). Cytotoxic activities of PM2, PM9-12 was compared to β,β-carotene and gallic acid against six human tumor cell cultures and three normal cell cultures (Table 2). The cytotoxic effect of PM compounds were in the same range but somewhat lower than that of gallic acid as control. The tumor-specific cytotoxicity as indicated by SI selective toxicity was only slightly observed in case of PM10 and PM11. The safety index (SI) values of PM10 and PM11 were 1.9 and 2.5, respectively (Table 2).

MDR reversal on tumor cells

MDR is a major problem in cancer treatment. The overexpression of drug transport proteins is a major mechanism for the MDR. In tumor cells overexpressing P-glycoprotein (P-gp), this results in reduced intracellular drug concentrations which in turn decrease the efficacy of a broad spectrum of some antitumor drugs. Molnár and coworkers have recently examined MDR modulating activity of several carotenoids and shown that capsanthin and capsorubin enhanced the drug accumulation most potently (Molnár *et al*., 2004). We measured the ability of PM9-10 and PM12 extracts to inhibit the P-gp mediated R-123 efflux in the MDR mouse T cell lymphoma L5178 transfected by human *MDR1* gene, which was cultured in colchicine-containing medium. The ability of PM9, 10 and 12 was studied on the drug accumulation of MDR tumor cells. These extracts showed dose dependent reversal of ABC transporter. Interestingly the β,β-carotene was more effective the same concentrations on the MDR cells, however these concentrations were toxic for the tested cells as the SSC and FSC values shows (Table 3).

Radical scavenging activity

Carotenoids are involved in the scavenging of two of reactive oxigen species (ROS), singlet molecular oxygen and peroxy radicals generated in the process of lipid peroxidation (Sies *et al*., 1993). At 400 μg/ml concentration PM9-10 and PM12 extracts showed lower singlet oxygen scavenging activity in comparison with the control β,β-carotene. In DPPH assay, the electron-donating capacity was strongest in the following order: PM10>PM9>PM12>β,β-carotene (Table 4).

CONCLUSION

Carotenoids are important dietary nutrients having antioxidant potential and are consequently being considered as important preventive strategic molecules. Antioxidants may protect against oxidative damages both by directly neutralizing reactive oxidants and by modulating gene expression contributing to oxidative stress (Silvaas *et al*., 2004). A mixture of carotenoids with different chemical structures and different antioxidants might be a promising agent for the medicinal use than individual carotenoids.

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