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Procyanidin Oligomers from Japanese Quince (*Chaenomeles japonica*) Fruit Inhibit Activity of MMP-2 and MMP-9 Metalloproteinases

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The influence of procyanidin extract from Japanese quince fruit on the activities of matrix metalloproteinases MMP-2 and MMP-9 secreted to culture medium by human peripheral blood mononuclear cells (PBMC) and by human leukemia HL-60 cells was investigated by gelatin zymography. The extract proved to be an effective inhibitor of the enzymes activities (for MMP-2 and MMP-9 secreted by PBMC $IC_{50} = 16-19$ μg extract/mL and 22−25 μg extract/mL, respectively). To identify the most effective components of the extract it was fractionated by means of column chromatography on TSKgel Toyopearl HW-40 (S) bed. The obtained fractions were analyzed by TLC, HPLC, and MALDI-TOF MS. Their antioxidant activity was measured as cation radicals $ABTS^{\bullet+}$ scavenging efficiency. The fractions VIII−XIV containing oligomers from trimer to hexamer (and probably higher oligomers) appeared to be the most effective inhibitors of MMP-2 and MMP-9 activity (IC_{50} value close to 4.6 μg total polyphenols/mL). To the best of our knowledge, it is the first report on gelatinase-inhibitory activity of Japanese quince fruit polyphenol extract. We conclude that polyphenols from Japanese quince can be used in cancer chemoprevention, although further studies are needed to elucidate the mechanisms underlying their biological activities.

KEYWORDS: Polyphenols; procyanidin oligomers; Japanese quince (*Chaenomeles japonica*); MMP-2; MMP-9; HL-60

INTRODUCTION

Proanthocyanidins, which are also known as condensed tannins, have been found in a range of fruits such as grapes, apples, and quince, beverages such as red wine, and foods such as black bean and cocoa (*1*−*8*). They have been reported to act as antioxidant and anticancer agents as well as to inhibit some enzymes and receptors (*2*−*11*). Among numerous anticancer activities proanthocyanidins can downregulate matrix metalloproteinases, thus blocking or slowing down angiogenesis, tumor growth, and metastasis (*12*).

Matrix metalloproteinases (MMPs) are a family of over 20 structurally related Zn^{2+}-dependent endopeptidases that are collectively able to degrade most extracellular matrix (ECM) components (*13*). Through modulation of ECM structure and composition as well as via proteolysis of non-ECM components (such as cytokines, chemokines, growth factor receptors, and cell adhesion molecules) MMPs are involved in a number of both physiological and pathological processes, including inflammation, angiogenesis, tumor growth, and metastasis. MMP-2 and -9 (type IV collagenases; gelatinases) degrade type IV collagen—the main component of basement membranes. Recent studies have demonstrated that polyphenols not only downregulate gelatinase expression (*2*) but also directly inhibit their activities (*14*). Among the most effective polyphenolic inhibitors of MMP activity one can list green tea polyphenols (e.g., epigallocatechin gallate with IC_{50} values for MMP-2 and MMP-9 close to 10 μM) (*14*). Oligomeric procyanidins from elm cortex were also reported as inhibitors of MMP-2 and MMP-9 (*15*). Our aim was to determine gelatinase-inhibitory activity of oligomeric procyanidins from Japanese quince (*Chaenomeles japonica*) fruit extract and its fractions.

MATERIALS AND METHODS

Materials. Ripe fruit (3 kg) of Japanese quince (*Chaenomeles japonica* L., *Rosaceae*) was collected from three bushes planted in a yard in the central region of Poland and stored at −20 °C before procyanidin extraction. All reagents used in our study were special grade

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Table 1. Characteristics of Japanese Quince Fruit Extract Fractions

fraction	polyphenol content [mg/mL]	flavanol content [mg/mL]	polyphenol content according to HPLC analysis [mg/mL] 280 nm	320 nm	360 nm	relative DP	polyphenol profile according to TLC analysis	oligomer identified by MALDI-TOF MS[a] analysis
I	0.00	0.00	–	–	–	–	–	–
II	0.04 ± 0.00	0.00	0.04	0.05	–	–	–	monomer (289)[a]
III	0.33 ± 0.04	0.00	0.37	0.10	0.01	–	phenolic acids	monomer (289)[a]
IV	1.56 ± 0.15	1.16 ± 0.02	1.81	0.00	0.04	1.6	dimer or monomer	monomer (289)[a]
V	2.04 ± 0.14	1.23 ± 0.10	2.13	–	0.02	3.3	two dimers	dimer (602)[b]
VI	0.25 ± 0.00	0.14 ± 0.00	0.22	–	–	2.5	dimer	dimer (602)[b]
VII	1.02 ± 0.09	0.60 ± 0.00	1.03	–	–	3.0	two trimers	dimer[c] (602)[b] and trimer (891)[b]
VIII	0.66 ± 0.06	0.37 ± 0.02	0.48	–	–	3.1	two trimers and tetramer[c]	trimer (891)[b]
IX	0.66 ± 0.07	0.36 ± 0.00	0.60	–	–	3.4	trimer[c] and tetramer	trimer[c] (891)[b] and tetramer (1179)[b]
X	0.66 ± 0.07	0.41 ± 0.03	0.78	–	–	3.6	tetramer	tetramer (1179)[b]
XI	0.54 ± 0.09	0.29 ± 0.03	0.72	–	–	3.6	tetramer[c] and pentamer	tetramer[c] (1179)[b] and pentamer (1467)[b]
XII	0.66 ± 0.04	0.39 ± 0.06	0.76	–	–	3.6	not well-separated pentamers	pentamer (1467)[b]
XIII	0.23 ± 0.00	0.10 ± 0.00	0.51	–	–	4.2	pentamer[c] and hexamer	pentamer (1467)[b] and hexamer (1756)[b]
XIV	1.99 ± 0.13	0.86 ± 0.06	2.41	–	–	3.8	higher oligomers	–

[a] MW given as $[M + H^+]$. [b] MW given as $[M + Na^+]$. [c] Low quantity of the oligomer.

commercial products purchased from Sigma-Aldrich (Saint Louis, MO), unless otherwise stated.

Preparation of Polyphenol-Rich Extract from Japanese Quince. Japanese quince extract was obtained according to the procedure of Oszmianski (*16*). Briefly, fresh pulp obtained from 1 kg of fruit without seeds was extracted twice with 90% acetone (in the ratio of 1:2.5 w/v), and then the pooled extracts were treated twice with trichloromethane (in the ratio of 1:1 v/v) to remove lipids. Proanthocyanidin oligomers were extracted from the water phase with ethyl acetate, and the resultant solutions were concentrated under vacuum and precipitated from trichloromethane.

The samples were stored at −20 °C prior to further analyses. The proanthocyanidin extracts were characterized in terms of total polyphenol content expressed as (+)-catechin equivalents (*17*), total flavanol content expressed as (+)-catechin equivalents (*18*), and relative degree of polymerization (DP) (*19*).

Fractionation of Condensed Tannins from Japanese Quince. Fractionation of condensed tannins from Japanese quince extract was performed by column chromatography on TSKgel Toyopearl HW-40 (S) support (Tosoh Corp., Tokyo, Japan) using methanol as an eluent (fractions I− XIII) and finally 80% aqueous solution of acetone (fraction XIV containing high molecular weight oligomers) (*20*). The fractions were concentrated till dryness and then dissolved in methanol. Each of the fractions was characterized in terms of total polyphenol content (*17*), total flavanol content (*18*), and relative degree of polymerization (*19*) (see **Table 1**). The fractions were also analyzed using thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). TLC was performed on a silica gel 60 plate (Merck, Darmstadt, Germany) with the use of a solution of formic acid, acetone and toluene (1:3:3; v/v/v) as a mobile phase (*21*).

HPLC Analysis. Phenolics were analyzed by reversed-phase (RP)-HPLC with the use of a Eurospher-100 C_{18} column (5 μm, 250 × 4.6 mm) (Knauer, Berlin, Germany). Binary mobile phase was prepared according to Dyrby et al. (*22*). Solvent A: water and formic acid in the ratio of 90:10 (v/v), respectively. Solvent B: water, acetonitrile, and formic acid in the ratio of 40:50:10 (v/v/v), respectively. The separation of phenolics was performed using the following gradient program with a flow rate of 1 mL/min: 0 min, 88% A + 12% B; 26 min, 70% A + 30% B; 40−43 min, 0% A + 100% B; 48−50 min, 88% A + 12% B.

The phenolics were divided into three groups on the basis of the wavelength at which the maximum of UV−vis absorption was observed. Flavan-3-ols and hydroxybenzoic acid derivatives were quantified at 280 nm and expressed as (+)-catechin equivalents, hydroxycinnamic acid derivatives at 320 nm as chlorogenic acid equivalents, and flavonols at 360 nm as rutin equivalents.

MALDI-TOF Analysis. Mass spectrometry analyses were performed using a Voyager-Elite MALDI-TOF mass spectrometer (Per-Septive Biosystems Inc., Framingham, MA) equipped with delayed extraction. Typical conditions included 20 kV acceleration voltage and nitrogen laser pulse (wavelength 337 nm). High-resolution negative-ion spectra were recorded in reflector mode. 2,5-Dihydroxybenzoic acid was used as a matrix.

Antioxidant Activity Measurement. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) radical cation scavenging activity was determined following the procedure described by Re et al. (*23*). $ABTS^{\bullet+}$ was produced by mixing 7 mmol/L ABTS water solution and 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand before use for 12−16 h in the dark at room temperature. The 70% ethanolic extract from the Japanese quince fruit or the fractions dissolved in methanol (or 80% aqueous solution of acetone − the fraction XIV) (20 μL) were mixed with 1 mL of diluted $ABTS^{\bullet+}$ solution, and their absorptions were measured at 734 nm after 6 min of incubation at 30 °C. Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) was used as a standard, and the capacity of free radical scavenging was expressed in micromoles of Trolox per 1 mg of total polyphenols.

HL-60 Cell Culture. HL-60 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 at an initial concentration of 2×10^4 per 200 μL in RPMI 1640 culture medium containing 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL fungizone, and 15% fetal bovine serum (FBS), replaced with fresh medium every 48 h. All components of the medium were purchased from Gibco BRL (Gaithersburg, MA). All experiments were carried out between passage 7 and 20. Cell viability was assessed on the basis of trypan blue exclusion.

Isolation and Culture of Human Peripheral Blood Mononuclear Cells (PBMC). Peripheral blood was diluted with PBS in the ratio of 1:1 and layered onto Histopaque-1077. After centrifugation at 400*g* for 30 min at room temperature, the layer of mononuclear cells was collected, and the cells were washed twice with PBS and suspended in the culture medium of the composition given above except for FBS content (10%). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 at an initial concentration of 2×10^5 per 200 μL.

Gelatin Zymography and Quantitative Analysis of Type IV Collagenase Activities. PBMC- and HL-60-conditioned media were used as sources of MMP-2 and MMP-9. For this purpose, cells in

exponential growth phase were resuspended at a concentration of 2×10^5 per 200 μL (PBMC) or 2×10^4 per 200 μL (HL-60) in RPMI 1640 medium supplemented with 2% FBS and distributed on 96-well plates. After 48 h the cells were spun down, and the obtained media were used as the sources of MMP-2 and MMP-9. Zymographic detection of type IV collagenases was carried out as previously described (*24*). Briefly, the same volumes of the media (usually 5 μL) were mixed with electrophoresis sample buffer containing sodium dodecyl sulfate (SDS), and the resultant mixtures were subjected to electrophoresis in a 10% polyacrylamide gel copolymerized with 1.5 mg/mL gelatin in the absence of β-mercaptoethanol. After electrophoresis the enzymes were renatured by incubation of the gel strips with 2.5% Triton X-100. The polyphenol extract was dissolved in 70% ethanol while the fractions were dissolved in methanol. Different volumes of these solutions (usually several microlitres) were added to the gelatinase-containing strips immersed in incubation buffer. After 18 h of incubation at 37 °C, the gels were stained for 1.5 h with 0.0125% Amido Black in a solution of acetic acid (7%) and ethanol (20%). Type IV collagenases were visualized without destaining as transparent bands against the dark blue background of Amido Black-stained slab gels. The zymographic gels were photographed with an ImageMaster VDS apparatus (Pharmacia Biotech, Uppsala, Sweden), and their densitometry was carried out using an ImageMaster VDS software (version 2.0). Within a certain range of enzymatic activity there is a linear dependence between MMP activity and integrated optical density of zymographic bands. In order to establish the linear range of relationships between the activities of MMPs and integrated optical density (IOD) of the lysis bands, zymograms containing increasing activities of commercial MMP-2 and MMP-9 (Boehringer Mannheim) were analyzed previously (*24*). We operated within the range in question.

Apoptosis Assay. Apoptosis was assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining. HL-60 cells were suspended in the culture medium (of the composition given above) and distributed on 24-well plates at a concentration of 1×10^5 per 1 mL. Incubation with the investigated extract, EGCG, or camptothecin lasted 24, 48, or 72 h at 37 °C. At these time points the cells were harvested, washed with phosphate buffered saline (PBS), and fixed (first with 1% paraformaldehyde solution in PBS and then with 70% ethanol solution) on ice in the dark. The fixed cells were stained with 1 μg/mL DAPI on ice in the dark for 30 min; then the stained cells were placed on glass slides, covered with mounting medium, and visualized under Olympus CKX41 fluorescence microscope at the magnification of 400×. Apoptotic cells were identified on the basis of nuclear morphology changes. At least 200 cells were counted for each preparation.

Statistical Evaluation of Data. Data is represented as mean ± standard deviation (SD). For each variable, three independent experiments were carried out, and within each experiment assays were performed in triplicate. The mean and SD values were calculated, and statistical calculations (*t* test) were performed using the Statgraphics Plus v. 2.1 software (Statistical Graphics, USA). *P* values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Characteristics of Japanese Quince Procyanidin Extract and Its Fractions. As unfractionated Japanese quince extract proved to be an effective gelatinase inhibitor (**Figure 1**), a more detailed analysis thereof was carried out. It was estimated that the extract contained 874.0 mg of total polyphenols/g and that flavanols constituted 38% of the preparation. MALDI-TOF MS analysis showed that the molecular weight distribution of the extract components ranged from 332 ($M + K^+$) to 2626 ($M + Na^+$) (**Figure 2**). These data indicate that the extract contains procyanidin monomers and oligomers (from dimers to nonamers). To identify the most active components of the Japanese quince extract, we carried out its fractionation on TSKgel Toyopearl HW-40 (S) bed. The fraction characteristics are given in **Table 1**. Fractions I and II were omitted from further bioassays because of none or very low polyphenol content,

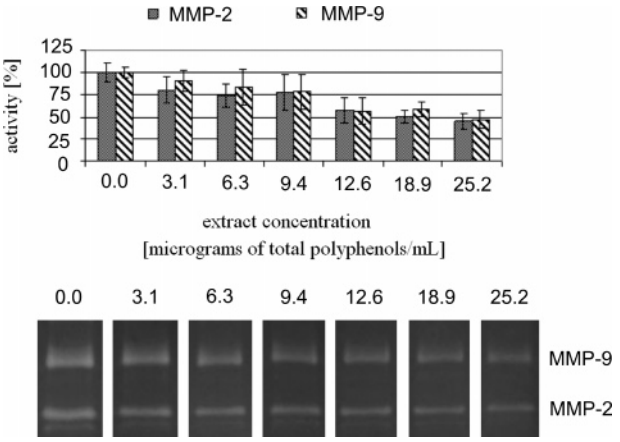
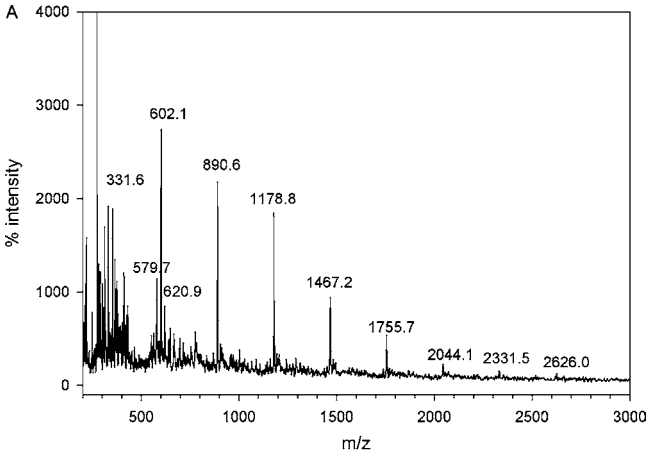


Figure 1. Influence of unfractionated Japanese quince fruit extract on type IV collagenase activities, as assessed by gelatin zymography. The same volume (5 μL) of PBMC-conditioned medium from the 48 h cell culture was loaded on each lane of the gel, and zymography was carried out as described in Materials and Methods.



B

Tentatively identified	M + H$^+$ (*)	M + Na$^+$	M + K$^+$
monomer	-	-	332
dimer	580	602	621
trimer	-	891	906
tetramer	-	1179	-
pentamer	-	1467	1483
hexamer	-	1756	1772
heptamer	-	2044	-
octamer	-	2332	-
nonamer	-	2626	-

(*) Peaks corresponding to the M+Na$^+$ forms of the oligomers dominated at all the MS spectra obtained but the peaks corresponding to the M+H$^+$ and/or M+K$^+$ forms of the oligomers were also present at the MS spectra of some fractions (data not shown).

Figure 2. MALDI-TOF mass spectrum of the Japanese quince extract (**A**) and the *m*/*z* values for the oligomeric procyanidins of the extract (**B**).

respectively. In order to characterize polyphenolic compounds present in the Japanese quince extract fractions, we carried out TLC on a silica gel plate (**Figure 3**). Phenolic acids are the sole constituents of fraction III whereas other fractions (from IV to XIV) contain only condensed tannins. The relative R_f values obtained for these compounds are slightly different from the literature data (*21*). The main constituents in the separated fractions are usually accompanied with small amounts of their isomers or other oligomers composed of flavanol units (catechin or epicatechin). The relative degree of polymerization was estimated by means of the chemical method (*19*) and, independently, by means of TLC (**Figure 3**, **Table 1**). On the basis of UV−vis maximum absorption, Japanese quince phenolics were qualified into one of the following subclasses: flavan-3-ols and

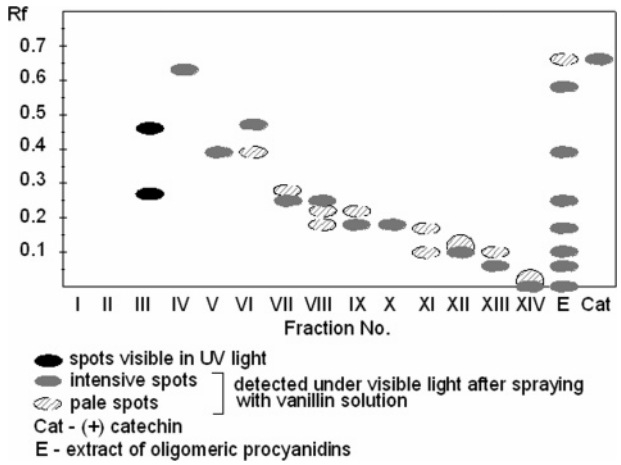


Figure 3. TLC analysis of oligomeric procyanidin preparation from Japanese quince fruit.

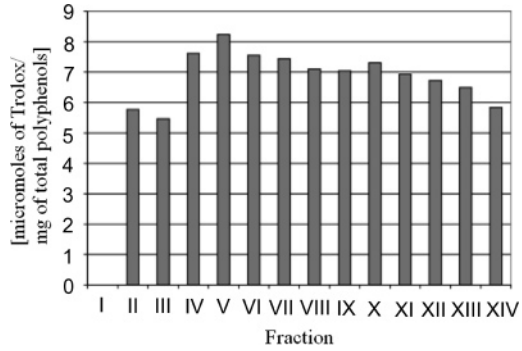


Figure 4. Antioxidant activity of the Japanese quince fruit extract fractions. ABTS radical cation scavenging activity is expressed as micromoles of Trolox per milligram of total polyphenols.

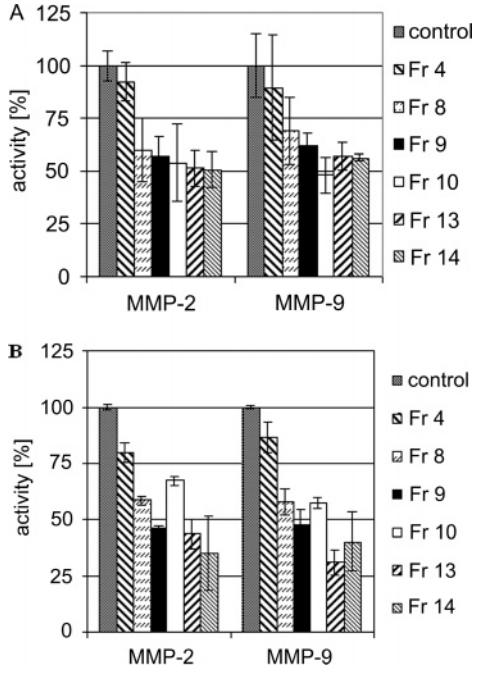


Figure 5. Influence of the selected fractions of Japanese quince fruit extract at a concentration of 4.6 μg total polyphenols/mL on MMP-2 and MMP-9 activity. PBMC-conditioned medium (**A**) and HL-60-conditioned medium (**B**) were used as sources of type IV collagenases.

hydroxybenzoic acid derivatives (280 nm), hydroxycinnamic acid derivatives (320 nm), and flavonols (360 nm) (**Table 1**).

The Japanese quince extract fractions were also qualitatively analyzed by MALDI-TOF MS (**Table 1**). In general, the MALDI-TOF mass spectra reveal the presence of usually one (sometimes two) oligomer(s) in each of the fractions studied. The results of TLC and MALDI-TOF MS analyses are slightly different (for example, according to MALDI-TOF MS, fraction II contains the monomer, while the TLC plate does not present any spot which would confirm that conclusion). Additional experiments should be performed on Japanese quince condensed tannins (such as nuclear magnetic resonance or thiolysis) to identify those compounds.

The antioxidant properties of the fractions were measured as cation radicals $ABTS^{\bullet+}$ scavenging efficiency, and the results indicate that fractions IV−X exhibited slightly higher antioxidant activity than the others, but no correlation between the degree of polymerization and antioxidant activity could be observed (**Figure 4**).

Inhibition of Type IV Collagenase Activities by Japanese Quince Oligomeric Procyanidins. Taking into account that inhibition of type IV collagenases by epigallocatechin gallate is well documented, we assessed anti-MMP activity of the Japanese quince polyphenols using them at concentrations close to the IC_{50} values previously reported for EGCG. According to Garbisa et al. (*14*), EGCG very effectively inhibited MMP-2 and -9 with IC_{50} of 8 μM and 13 μM, respectively. Under our experimental conditions, IC_{50} values for EGCG were lower for both gelatinases, namely 2.5−5.0 μM for MMP-2 and 5.0−7.5 μM for MMP-9 (data not shown). The influence of the Japanese quince extract fractions on gelatinase activities was determined at 4.6 μg total polyphenols/mL (**Figure 5**), which corresponds to a 10 μM concentration of EGCG. Under these conditions, fractions VIII−XIV containing oligomers from trimer to hexamer (and probably higher oligomers) appeared to be the most effective inhibitors of MMP-2 and MMP-9 activity (IC_{50} value close to 4.6 μg total polyphenols/mL). The inhibition was slightly higher in the case of the enzymes secreted by HL-60 cells as compared to those secreted by PBMC (**Figure 5**). To the best of our knowledge, it is the first report on gelatinase-inhibitory activity of Japanese quince fruit polyphenol extract. Thus far, a related study was carried out on Chinese quince (*Chaenomeles sinensis*) fruit extract (*25*). Only a very slight inhibition of MMP-9 activity (ranging from 1.2% to 5.6%) was observed for Chinese quince fruit extracts obtained with the use of several solvents. However, no data regarding the phenolic profile of Chinese quince fruit extract was given in the work in question.

Our results are in accordance with other data on biological activities of oligomeric procyanidins. Song et al. investigated elm cortex extract as a potential therapeutic agent against periodontal diseases (*15*). The crude extract and procyanidin oligomers isolated from this extract (average DP = 5.3) inhibited MMP-2 with IC_{50} of 45 and 33 μg/mL, respectively. It is important to note that there are reports on other biological activities of procyanidin oligomers with DP close to 5. Black bean condensed tannins (a mixture of trimers and tetramers) were demonstrated to inhibit colon, breast, and prostate cancer cell proliferation (*6*). Pentameric procyanidins from *Theobroma cacao* seeds not only significantly downregulated ErbB2 tyrosine kinase in human aortic endothelial cells (*7*) but also selectively inhibited the growth of human breast cancer cells (*8*). Oligomers with DP > 3 were indicated as possibly responsible for the direct inhibition of xanthine oxidase activity by French maritime pine (*Pinus maritima*) bark extract (Pycnogenol) in which 75% of

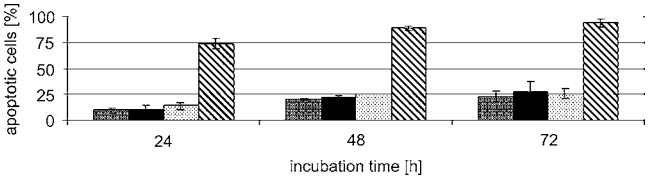


Figure 6. Evaluation of apoptosis-inducing ability of the Japanese quince extract on the basis of cell count after staining with DAPI. From left to right the bars correspond to the following: control, EGCG (45.8 μg/mL = 100 μM), Japanese quince extract (45.8 μg total polyphenols/mL), and camptothecin (2 μM).

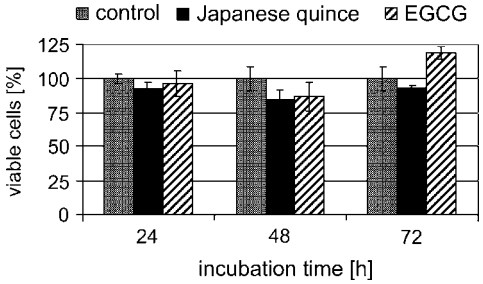


Figure 7. Influence of the Japanese quince extract (45.8 μg total polyphenols/mL) and EGCG (45.8 μg/mL = 100 μM) on HL-60 cell viability, as estimated on the basis of cell count after staining with trypan blue.

procyanidins range from monomer to heptamer (*10*). *Pinus maritima* bark extract was also shown to inhibit the activity of phosporylase kinase, protein kinase C and protein kinase A (*11*). Apple procyanidins (from trimers to pentamers) inhibited melanogenesis in B16 mouse melanoma cells (*4*).

The reasons for which oligomeric procyanidins interact with the above-mentioned proteins are not presently known. Baxter et al. demonstrated that in the case of a proline-rich protein fragment proline residues followed by prolines are geometrically preferred polyphenol binding sites, and their interaction consists of a face-to-face stacking of aromatic groups onto proline residues (*26*). It is known that MMP-9 (GI:116863) contains a so-called proline-rich insert bearing several proline dimers, but MMP-2 (GI:11342666) sequence contains only one such a dimer. On the other hand, our results clearly demonstrate that both enzymes were inhibited to a similar extent by the procyanidin-rich Japanese quince extract. Therefore, one can suggest that the interactions between oligomeric procyanidins and gelatinases depend not only on the proline content but also on the amino acid sequences of these enzymes.

Baxter et al. suggested that the mode of interaction of polyphenols with globular proteins might involve surface-exposed aromatic residues (*26*). This suggestion is supported by the results of Ottaviani et al. who showed that procyanidin hexamer most effectively quenched tryptophane residues on the surface of angiotensin I converting enzyme (ACE), whereas tetramer was the strongest inhibitor of ACE activity (*27*). Recently Kondo et al. calculated a global minimum-energy conformation of an oligomeric procyanidin and suggested that procyanidins with a mean degree of polymerization of 7 are likely to form helical structures with externally oriented B-rings which may interact with hydrophobic amino acid residues (*28*). The ability of assuming a helical shape further distinguishes oligomeric procyanidin molecules from monomers and dimers in terms of possible biological activities.

Apoptosis Studies. Proapoptotic activities of condensed tannins of different origin have been reported for different tumor cell lines. Apple procyanidins induced apoptotic cell death in human colon cancer-derived metastatic SW620 cells (*3*). Taking into account these studies, we carried out preliminary experiments in order to estimate the proapoptotic activity of the Japanese quince polyphenol extract toward HL-60 cells. In control experiments we used camptothecin at a 2 μM concentration as an apoptosis inducer and EGCG at a 100 μM (45.8 μg/mL) concentration as a reference polyphenol. Apoptotic cells were identified on the basis of DAPI staining which allows the visualization of apoptotic bodies arising after DNA fragmentation and chromatin condensation at the periphery of a nuclear membrane. After 24 h of incubation, camptothecin induced apoptosis of 74% of the HL-60 cells (**Figure 6**), while neither EGCG nor the Japanese quince polyphenol extract used at the concentration corresponding to 100 μM EGCG triggered apoptosis of the cells to a level significantly higher than that one in the control samples.

Furthermore, we assessed the influence of Japanese quince condensed tannins on HL-60 cells viability. At a concentration of 45.8 μg total polyphenols/mL and after 24, 48, or 72 h of incubation, neither the investigated extract nor EGCG exerted a marked influence on HL-60 viability, as estimated by cell count after staining with trypan blue (**Figure 7**).

Recently Shoji et al. reported that, after the administration of apple condensed tannins to rats, free procyanidins (up to pentamers) were detected in plasma, which might imply their involvement in physiological functions (*29*). However, the tested compounds did not come into a contact with salivary proteins because of the fact that they were injected via direct stomach intubation. Moreover, the animals were starved for 15 h prior to the experiment; hence the condensed tannins did not come into the contact with any food components. Studies on procyanidin bioavailability in humans were carried out, among others, by Holt et al., who demonstrated that after consumption of a flavanol-rich cocoa beverage only procyanidin dimer and monomers epicatechin and catechin were absorbed into the circulation (*30*). We report that condensed tannins from Japanese quince fruit significantly inhibit MMP-2 and -9 activities, which renders them promising chemopreventive agents, although more data on procyanidin bioavailability in humans is needed in order to estimate their possible health effects in vivo.

ABBREVIATIONS USED

DAPI, 4′,6-diamidino-2-phenylindole; DP, degree of polymerization; EGCG, (−)-epigallocatechin-3-gallate; HL-60, human promyelocytic leukemia cells; HPLC, high-performance liquid chromatography; GI, gene identifier; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MMP, matrix metalloproteinase; PBMC, peripheral blood mononuclear cells; TLC, thin layer chromatography.

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LITERATURE CITED

(1) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.

(2) Vayalil, P. K.; Mittal, A.; Katiyar, S. K. Proanthocyanidins from grape seeds inhibit expression of matrix metalloproteinases in human prostate carcinoma cells, which is associated with the inhibition of activation of MAPK and NFkappaB. *Carcinogenesis* **2004**, *25*, 987−995.

(3) Gosse, F.; Guyot, S.; Roussi, S.; Lobstein, A.; Fischer, B.; Seiler, N.; Raul, F. Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis* **2005**, *26*, 1291−1295.

(4) Shoji, T.; Masumoto, S.; Moriichi, N.; Kobori, M.; Kanda, T.; Shinmoto, H.; Tsushida, T. Procyanidin trimers to pentamers fractionated from apple inhibit melanogenesis in B16 mouse melanoma cells. *J. Agric. Food Chem.* **2005**, *53*, 6105−6111.

(5) Silva, B. M.; Andrade, P. B.; Mendes, G. C.; Valentao, P.; Seabra, R. M.; Ferreira, M. A. Analysis of phenolic compounds in the evaluation of commercial quince jam authenticity. *J. Agric. Food Chem.* **2000**, *48*, 2853−2857.

(6) Bawadi, H. A.; Bansode, R. R.; Trappey, A., II; Truax, R. E.; Losso, J. N. Inhibition of Caco-2 colon, MCF-7 and Hs578T breast, and DU 145 prostatic cancer cell proliferation by water-soluble black bean condensed tannins. *Cancer Lett.* **2005**, *218*, 153−162.

(7) Kenny, T. P.; Keen, C. L.; Jones, P.; Kung, H. J.; Schmitz, H. H.; Gershwin, M. E. Pentameric procyanidins isolated from *Theobroma cacao* seeds selectively downregulate ErbB2 in human aortic endothelial cells. *Exp. Biol. Med. (Maywood)* **2004**, *229*, 255−263.

(8) Ramljak, D.; Romanczyk, L. J.; Metheny-Barlow, L. J.; Thompson, N.; Knezevic, V.; Galperin, M.; Ramesh, A.; Dickson, R. B. Pentameric procyanidin from *Theobroma cacao* selectively inhibits growth of human breast cancer cells. *Mol. Cancer Ther.* **2005**, *4*, 537−546.

(9) Faria, A.; Calhau, C.; de Freitas, V.; Mateus, N. Procyanidins as antioxidants and tumor cell growth modulators. *J. Agric. Food Chem.* **2006**, *54*, 2392−2397.

(10) Moini, H.; Guo, Q.; Packer, L. Enzyme inhibition and protein-binding action of the procyanidin-rich French maritime pine bark extract, pycnogenol: effect on xanthine oxidase. *J. Agric. Food Chem.* **2000**, *48*, 5630−5639.

(11) Nardini, M.; Scaccini, C.; Packer, L.; Virgili, F. *In vitro* inhibition of the activity of phosporylase kinase, protein kinase C and protein kinase A by caffeic acid and a procyanidin-rich pine bark (*Pinus maritima*) extract. *Biochim. Biophys. Acta* **2000**, *1474*, 219−225.

(12) Katiyar, S. K. Matrix metalloproteinases in cancer metastasis: molecular targets for prostate cancer prevention by green tea polyphenols and grape seed proanthocyanidins. *Endocr. Metab. Immune Disord. Drug Targets* **2006**, *6*, 17−24.

(13) Chang, C.; Werb, Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis, and metastasis. *Trends Cell Biol.* **2001**, *11*, S37−43.

(14) Garbisa, S.; Sartor, L.; Biggin, S.; Salvato, B.; Benelli, R.; Albini, A. Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. *Cancer* **2001**, *91*, 822−832.

(15) Song, S. E.; Choi, B. K.; Kim, S. N.; Yoo, Y. J.; Kim, M. M.; Park, S. K.; Roh, S. S.; Kim, C. K. Inhibitory effect of procyanidin oligomer from elm cortex on the matrix metalloproteinases and proteases of periodontopathogens. *J. Periodontal Res.* **2003**, *38*, 282−289.

(16) Oszmianski, J. Sposob otrzymywania aktywnych biologicznie oligomerow proantocyjanidyn z surowcow roslinnych (Method of preparation of biologically active oligomeric proanthocyanidins from plant raw materials). Polish patent No. P-296521, 1992.

(17) Peri, C.; Pompei, G. An assay of different phenolic fractions in wines. *Am. J. Enol. Vitic.* **1971**, *22*, 55−57.

(18) Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica* II. *J. Sci. Food Agric.* **1959**, *10*, 63−68.

(19) Oszmianski, J.; Bourzeix, M. Comparison of methods for determining the content and polymerization of proanthocyanidins and catechins. *Pol. J. Food Nutr. Sci.* **1996**, *46* (5), 43−50.

(20) Oszmianski, J; Bourzeix, M. Preparation of catechin and procyanidin standards from hawthorn (*Crataegusa azarolus* L.) and pine (*Pinus mesogeensis fieschi*) barks. *Pol. J. Food Nutr. Sci.* **1995**, *4/45* (2), 89−96.

(21) Lea, A. G. H.; Bridle, P.; Timberlake, C. F.; Singleton, V. L. The procyanidins of white grapes and wines. *Am. J. Enol. Vitic.* **1979**, *30*, 289−300.

(22) Dyrby, M.; Westergaard, N.; Stapelfeldt, H. Light and heat sensitivity of red cabbage extract in soft drink model systems. *Food Chem.* **2001**, *72*, 431−437.

(23) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radicals Biol. Med.* **1999**, *26*, 1231−1237.

(24) Hrabec, E.; Strek, M.; Nowak, D.; Greger, J.; Suwalski, M.; Hrabec, Z. Activity of type IV collagenases (MMP-2 and MMP-9) in primary pulmonary carcinomas: a quantitative analysis. *J. Cancer Res. Clin. Oncol.* **2002**, *128*, 197−204.

(25) Seo, U. K.; Lee, Y. J.; Kim, J. K.; Cha, B. Y.; Kim, D. W.; Nam, K. S.; Kim, C. H. Large-scale and effective screening of Korean medicinal plants for inhibitory activity on matrix metalloproteinase-9. *J. Ethnopharmacol.* **2005**, *97*, 101−106.

(26) Baxter, N. J.; Lilley, T. H.; Haslam, F.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.

(27) Ottaviani, J. I.; Actis-Goretta, L.; Villordo, J. J.; Fraga, C. G. Procyanidin structure defines the extent and specificity of angiotensin I converting enzyme inhibition. *Biochimie* **2006**, *88*, 359−365.

(28) Kondo, K.; Uchida, R.; Tokutake, S.; Maitania, T. Polymeric grape-seed procyanidins, but not monomeric catechins and oligomeric procyanidins, impair degranulation and membrane ruffling in RBL-2H3 cells. *Bioorg. Med. Chem.* **2006**, *14*, 641−649.

(29) Shoji, T.; Masumoto, S.; Moriichi, N.; Akiyama, H.; Kanda, T.; Ohtake, Y.; Goda, Y. Apple procyanidin oligomers absorption in rats after oral administration: analysis of proyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *J. Agric. Food Chem.* **2006**, *54*, 884−892.

(30) Holt, R. R.; Lazarus, S. A.; Sullards, M. C.; Zhu, Q. Y.; Schramm, D. D.; Hammerstone, J. F.; Fraga, C. G.; Schmitz, H. H.; Keen, C. L. Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *Am. J. Clin. Nutr.* **2002**, *76*, 798−804.

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