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Structure Activity Relationship of Antioxidative Property of Flavonoids and Inhibitory Effect on Matrix Metalloproteinase Activity in UVA-Irradiated Human Dermal Fibroblast

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Collagenase, a matrix metalloproteinases (MMPs), is a key regulator in the photoaging process of skin due to the reactive oxygen species generated after exposure to ultraviolet A (UVA). Flavonoid compounds have been demonstrated to possess antioxidant properties, and could be useful in the prevention of photoaging. In this study, to investigate the structure-activity relationship of flavonoid compounds on their antioxidant property and inhibitory effects against the MMP activity, the effects of several flavonoids; myricetin, quercetin, kaempferol, luteolin, apigenin and chrysin, on the reactive oxygen species scavengering activity and inhibitory effect against the MMP activity were examined *in vitro* and in human dermal fibroblasts induced by UVA. The relative order of antioxidative efficacy, as determined using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method and the xanthine/xanthine oxidase system, was as follows; flavones: luteolin > apigenin > chrysin, flavonols: myricetin > quercetin > kaempferol, and correlated with the respective number of OH group on their B-ring. In good correlation with the antioxidant properties, the flavonoids inhibited the collagenase activities, in a dose-dependent manner, and the MMP expression. These results suggested the UVA induced antioxidative activity and inhibitory effects of flavonoids on the collagenase in human dermal fibroblasts depends on the number of OH group in the flavonoid structure, and those with a higher number of OH group may be more useful in the prevention of UV stressed skin aging.

Key words: Flavonoids, Antioxidant, MMP, Structure activity relationship, Human skin fibroblast

INTRODUCTION

The skin is the organ most susceptible to damage by ultraviolet (UV) irradiation. Matrix metalloproteinases (MMPs) are a family of zinc- and calcium- dependent endoproteinases, which are capable of degrading almost all of the components of the extracellular matrix (ECM) of the skin (Chambers and Matrisian, 1997). MMPs were divides into subclasses, including collagenases, gelatinases, stromelysins, matrilysins, membrane type-MMPs (MT-MMPs) and other non-classified MMPs (Mignatti and Rifkin, 1993). MMPs are necessary for tissue remodeling and the healing cascade under normal physiological conditions. Clinically, naturally aged skin is smooth, pale and finely wrinkled; in contrast, photoaged skin is coarsely wrinkled (Gilchrest, 1989). Alterations of the ECM have been suggested as a cause of skin wrinkling and loss of elasticity, observed both in naturally and photoaged skin (Fisher *et al.*, 1996; Varani *et al.*, 2000). UV irradiation induces the synthesis of MMP in skin fibroblasts *in vitro* and MMP-mediated collagen destruction, an important component of the ECM (Fisher *et al.*, 1996).

Reactive oxygen species (ROS) are generated by UV-irradiation, which result in oxidative damage to the lipids, proteins and DNA of skin (Cunningham *et al.*, 1985; Hanson and Clegg, 2002, Vile and Tyrrell, 1995). The ROS produced by UV irradiation regulate gene expressions, including induction of MMPs through the redox-dependent activation of NF-κB (Shang *et al.*, 2002; Scharffetter-Kochanek *et al.*, 2000; Wenk *et al.*, 2001). Acute exposure of human

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skin *in vivo* to ultraviolet (UV) irradiation up-regulates the syntheses of several matrix metalloproteinases (MMPs), including MMP-1 (interstitial collagenase), MMP-3 (stromelysin-1) and MMP-9 (92-kd gelatinase B), which degrade skin collagen (Fisher *et al.*, 1996; Fisher *et al.*, 1997). Recent studies have demonstrated that certain MMP are highly induced in inflamed as well as photoaged skin, and breakdown the dermal matrix proteins, such as collagen and elastin; this possibly leads to prolonged skin damage and wrinkle formation (Fisher and Voorhees, 1998). Therefore, the agents that inhibit collagenase activity may have beneficial effects for maintaining healthy skin by preventing degradation of the dermal matrix.

Flavonoids are naturally occurring phenolic phytochemicals, which have been reported to possess several biological properties *in vitro*. Despite the similarity between flavonoid structures, the biological properties vary considerably with only minor modifications in their structure. The number and specific positions of hydroxyl groups and the nature of the substitutions determine whether flavonoids function as strong antioxidative (Rice-Evans *et al.*, 1996; Bors *et al.*, 1990), anti-inflammatory, antiproliferative (Sato *et al.*, 2002; Gamet-Payrastre *et al.*, 1999) or enzyme modulating agents. Hydroxylation of the B-ring, where a catechol group is the key to the flavonoid activity, coupled with a 2, 3 double bond in conjugation with a 4-oxo function (carbonyl group) in the C-ring (Rice-Evans *et al.*, 1996; Bors *et al.*, 1990). In this respect, the effects of flavonoids on MMP have also been previously examined. For instance, delphinidine and several other flavonoids have been reported to inhibit gelatinases (MMP-2 and MMP-9) (Nagase *et al.*, 1998; Ende and Gebhardt, 2004). However, the structural activity relationship of flavonoids on their antioxidative activity and MMP expression in UVA irradiated human dermal fibroblasts has not been fully reported. In the present study, the structural activity effects of the flavonoids; myricetin, quercetin, kaempferol, luteolin, apigenin and chrysin, on the antioxidative and MMP activities were investigated both *in vitro* and in human dermal fibroblasts.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Cambrex/BioWhittaker Inc. (Walkersville, MD, U.S.A.). 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium chloride (NBT), hypoxanthine, xanthine oxidase, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), chrysin, apigenin, luteolin, kaempherol, quercetin and myricetin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade and obtained from commercial suppliers.

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

The free radical scavenging activity of flavonoids was determined using DPPH, and measured according to the Blois' method (Blois, 1958), with minor modifications. A sample was dissolved in 0.1 mL dimethyl sulfoxide (DMSO) and then added to 0.1 mL of 0.1 mM DPPH in methanol. The mixture was shaken vigorously and allowed to stand for 10 min at room temperature in the dark. The absorbance at 565 nm due to DPPH was measured using a microplate spectrophotometer. The capability to scavenge the DPPH radical was calculated using the following equation: radical scavenging activity (%) = $[(OD_{control} - OD_{sample})/OD_{control}] \times 100$.

Superoxide anion radical scavenging activity

The scavenging potential for superoxide radicals was analyzed via a hypoxanthine/xanthine oxidase generating system, coupled with NBT reduction, following the method of Furuno (Furuno *et al.*, 2002). Xanthine (3 mM), EDTA (3 mM), NBT(0.72 mM) and flavonoids were added to 0.05 M Na_2CO_3 buffer (pH 10.2) and incubated for 10 min at 25°C. After incubation, 250 mU/mL xanthine oxidase was added to the reaction mixture and incubated at 25°C for a further 25 min. The absorption of the reaction mixture was monitored spectrophotometrically at 565 nm. The results were expressed as the percent inhibition.

MMP-1 activity

The *in vitro* collagenase inhibition assay, based on the fluorescence measurement of collagen fragments on cleavage by MMP-1, was performed using EnzChek Collagenase/Gelatinase kits (Molecular Probes Inc., OR, U.S.A.). The enzymes were mixed with quenched fluorescent substrates (25 μg/mL), in a final volume of 200 μL reaction buffer, in 96-well microplates. Digested products from DQ collagen substrates have absorption and emission maxima at 495 and 515nm, respectively, in a fluorescence microplate reader (Perkin Elmer, MA, U.S.A.). For all the MMPs tested, the activities under these conditions were linear for at least 15 min. For each time point, correction of the background fluorescence was achieved by subtracting the values derived from a no-enzyme control.

Cell culture

Human dermal fibroblasts from newborn foreskin were acquired from Modern Tissue Technologies (Seoul, Korea), and cultured on DMEM/Ham's F-12 nutrient mixture, containing 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 μg/mL), at 37°C in a humidified

atmosphere containing 5% CO_2. Human dermal fibroblasts subcultures were performed by trypsinisation, and used between the sixth and tenth passages.

UVA irradiation

Human dermal fibroblasts (1.5×10^5 cells/mL) were seeded into 35ø plates (Corning Inc., NY, U.S.A.) and cultured overnight. Prior to irradiation, when the cells were 80% confluent, they were washed twice with PBS. A UVA simulator (Jhonsam Inc., Seoul, Korea), filtered for UVA irradiation (320~400 nm), was used at a tube-to-target distance of 15 cm. The dose of UVA radiation was set at 6.3 J/cm^2, as determined using a UV radiometer (International Light Inc., MA, U.S.A.). During irradiation, control cells were treated identically, but without the exposure to UV light. After irradiation, fresh serum-free medium, containing flavonoids at various concentrations, was added to the cells at 37°C for 24 h.

Cell viability assay

The number of viable cells was determined by the ability of mitochondria to convert MTT (3(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to formazan dye. Human dermal fibroblasts were cultured overnight onto 96-well plate at a density of 1×10^4 cells/well. The cells were then treated with various reagents, according to the experimental design. The medium was removed after 24 h and 10 μL of 10 mg/mL MTT added to each well, and then incubated for a further 4 h in a 5% CO_2 humidified atmosphere at 37°C. The MTT was removed, the cells lysed with 100 μL DMSO, and the absorbance then measured at 570 nm using a microplate reader (ELX 800, Bio-Tek Instruments, VT, U.S.A.).

Enzyme-Linked Immunosorbent Assay (ELISA)

The level of MMP-1 expression was assayed using an enzyme-linked immunosorbent assay (ELISA). Human dermal fibroblasts (2×10^4 cells/well) were seeded into 48-well plates and cultured overnight. The culture media were replaced with DMEM/Ham's F-12 containing flavonoids at different concentrations. After 24 h incubation, the supernatants were transferred into a 96-well plate, and the same volume of coating buffer (Na_2CO_3 1.59%, $NaHCO_3$ 2.93%, NaN_3 0.20%, $MgCl_2$ 1.02%, pH 9.6) added, and incubated for a further 24 h. The supernatants were removed, and the coated well washed three times with PBS, containing 0.05% Tween 20 (PBST), followed by blocking with 3% bovine serum albumin (BSA) in PBS for 1 h at 37°C. After washing with PBST, 0.3 μg/mL of anti-MMP-1 monoclonal antibody in PBST was added into each well and incubated for 60 min. After washing the wells three times with PBST, 0.2 μg/mL of anti-mouse IgG conjugated with alkaline phosphatase in PBST, was added, and the mixture incubated for a further 60 min. After washing five times with PBST, 100 μL of 1 mg/mL p-nitrophenyl phosphate (pNPP) in a diethanolamine buffer was added. The optical density was measured at 405 nm after 30 min using an automated microplate reader.

Semiquantitative RT-PCR

The total RNA was isolated using RNeasy Mini Kit (Qiagen, MD, U.S.A.), according to the manufacturer's protocol. A volume containing 1 μg total RNA from each sample was subjected to reverse transcription, using Omniscript RT Kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR amplification was performed in a reaction volume of 25 μL of cDNA product and HotStarTaq™ DNA polymerase (Qiagen, Hilden, Germany), using an automatic heat-block DNA thermal cycler (ASTEC PC801, ASTEC Inc, Tokyo, Japan). Amplification of the constitutively expressed β-actin was used as an internal control to assess the reverse transcription efficiency. The oligonucleotide primers used were: 5'-AAA GGG AAT AAG TAC TGG GC-3' (sense) and 5'-AAT TCC AGG AAA GTC ATG TG-3' (antisense) for MMP-1; 5'-ATG CAG AAG GAG ATC ACT GC-3' (sense) and 5'-CTG CGC AAG TTA GGT TTT GT-3' (antisence) for β-actin. The temperature cycling conditions used for amplification were as follows: for MMP-1 and β-actin, 15 min at 94°C, then 28 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 60 s, and final extension at 72°C for 10 min. The amplification products were subjected to electrophoresis on 1.5% agarose gel, visualized by ethidium bromide staining, and then photographed. Gel images were scanned using an image analysis system (BIS303PC, DNR Imaging Systems Ltd, UK). The intensities of specific PCR bands were quantitated in relation to the â-actin bands amplified from the same cDNA, using the densitometric program (NIH Image software, MD, U.S.A.).

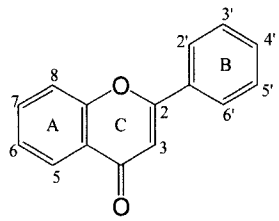
Statistical analysis

Statistical significance was determined using the Student's t-tests. Results are presented as the means± S.E.M. P values < 0.05 were regarded being significantly different.

RESULTS

Free radical scavenging activity of flavonoids

DPPH has been widely used to evaluate free radical generation (Cotelle *et al.*, 1996; Ozcelik *et al.*, 2003). In the DPPH assay, the antioxidants are able to reduce the stable DPPH radical to the yellow coloured diphenylpicrylhydrazine. The method is based on the reduction of an alcoholic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form, DPPH-H, during the reaction. To evaluate



Compound		Substituents							
		3	5	6	7	2'	3'	4'	5'
Flavones	Chrysin	H	OH	H	OH	H	H	H	H
	Apigenin	H	OH	H	OH	H	H	OH	H
	Luteolin	H	OH	H	OH	H	OH	OH	H
Flavonols	Kaempferol	OH	OH	H	OH	H	H	OH	H
	Quercetin	OH	OH	H	OH	H	OH	OH	H
	Myricetin	OH	OH	H	OH	H	OH	OH	OH

Fig. 1. The model flavonoids used in the present study.

the free radical scavenging activity of flavonoids, a DPPH free radical scavenging activity assay was performed. As shown in Table I, flavonoids scavenged the DPPH radical in a dose-dependent manner, and the DPPH radical scavenging activity (IC_{50}) was decreased in the following order: myricetin (4 μM) > quercetin (5 μM) = luteolin (5 μM) > kaempferol (12 μM) > chrysin > apigenin.

Scavenging activity of flavonoids against superoxide anion radical

The superoixde anion radical was generated in a hypoxanthine/xanthine oxidase system and assayed by the reduction of NBT. In this method, the superoxide anion reduces the yellow dye to produce the blue formazan, which can subsequently be measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the formation of the blue NBT (Cos *et al.*, 1997; Parejo *et al.*, 2002). Consistent with the order of inhibition of the free radical scavenging effect, flavonoids scavenged superoxide radicals in a dose-dependent manner, and the superoxide radical scavengering activity (IC_{50}) was decreased in the following order: myricetin (0.6 μM) > luteolin (0.8 μM) > kaempferol (0.20 μM) > quercetin (0.50 μM) > chrysin (100 μM) > apigenin (Table II).

Table I. Free radical scavenging activity of flavonoids

Compound		DPPH radical scavenging activity (%)[a]			IC_{50}
		0.01 mM	0.1 mM	1 mM	
Flavones	Chrysin	-[b]	6.4 ± 0.1	8.5 ± 0.2	-
	Apigenin	-	8.45 ± 0.3	10.2 ± 0.2	-
	Luteolin	52.9 ± 0.9	83.3 ± 1.6	96.2 ± 1.7	5 μM
Flavonols	Kaempferol	45.2 ± 0.7	94.3 ± 1.2	97.2 ± 1.9	12 μM
	Quercetin	76.7 ± 1.2	96.0 ± 1.6	98.1 ± 1.6	5 μM
	Myricetin	85.6 ± 1.1	92.8 ± 1.4	96.9 ± 1.9	4 μM

[a]Mean values of three measurements ± S.D.
[b]less than 5% DPPH radical scavenging activity

Inhibition of collagenase by flavonoids

The collagenase (MMP-1) activity was measured using fluorescein-conjugated collagen as the substrate, with collagenase purified from *Clostridium histolyticum* provided with the assay kit, which served as a control enzyme. This substrate is efficiently digested by collagenases to yield highly fluorescent peptides. The increase in fluorescence is proportional to proteolytic activity. The collagenase inhibitory effect of flavonoids was measured, with the flavonols found to be stronger inhibitors than the flavones, implying that the C-3-hydroxyl group in the compounds is important for higher inhibitory activity (apigenin vs. kaempferol and luteolin vs. quercetin). As shown in Table III, the collagenase activity (with same amount of flavonoid; 0.2 mM) was inhibited in the following order: myricetin (29.3%) > quercetin (21.1%) > kaempferol (12.5%) > apigenin (14.5%) > luteolin (11.1%) > chrysin (7.3%).

Effect of flavonoids on the cell viability of HDFs

To evaluate the effects of various flavonoids on the cell viability of HDFs, the cells were treated with different

Table II. Superoxide radical scavenging activity of flavonoids

Compound		Superoxide radical scavenging activity (%)[a]			IC_{50}
		0.001 mM	0.01 mM	0.1 mM	
Flavones	Chrysin	-[b]	41.0 ± 1.2	42.3 ± 1.9	100 μM
	Apigenin	-	14.3 ± 0.4	15.7 ± 0.2	-
	Luteolin	7.3 ± 0.4	66.4 ± 1.2	91.8 ± 1.0	8 μM
Flavonols	Kaempferol	-	41.4 ± 0.9	88.2 ± 0.9	20 μM
	Quercetin	8.64 ± 0.2	34.0 ± 0.7	54.1 ± 0.6	50 μM
	Myricetin	24.6 ± 0.9	79.5 ± 1.8	96.4 ± 1.5	6 μM

[a] Mean values of three measurements ± S.D.
[b] less than 5% superoxide radical scavenging activity

Table III. Inhibitory effect of flavonoids on collagenase

Compound		Collagenase inhibition (%)[a]		
		0.05 mM	0.1 mM	0.2 mM
Flavones	Chrysin	-[b]	-	7.3 ± 0.3
	Apigenin	6.2 ± .0.2	9.7 ± 0.4	14.5 ± 0.5
	Luteolin	-	5.9 ± 0.3	11.1 ± 0.8
Flavonols	Kaempferol	-	6.1 ± 0.4	12.5 ± 0.7
	Quercetin	7.5 ± 0.3	8.6 ± 0.4	21.1 ± 1.2
	Myricetin	-	12.7 ± 0.9	29.6 ± 1.2
1,10-Phenanthroline		18.9 ± 1.5	39.4 ± 2.6	75.1 ± 6.7

[a] Mean values of three measurements ± S.D.
[b] less than 5% collagenase inhibition

concentrations of several flavonoids, including chrysin, apigenin, luteolin, kaempherol, quercetin and myricetin. Flavonoids have been shown to reduce the cell viability of HDFs in a dose-dependent manner (Fig. 2). However, in the presence of up to 100 μM, the flavonoids showed no cytotoxicity toward HDFs.

Effect of flavonoids on the expression of MMP-1 in UV-irradiated cultured HDFs

UV irradiation damages human skin and causes premature skin aging (photoaging) through the activation of MMPs, which are responsible for the degradation of collagen, gelatin and other components of the ECM (Kligman, 1969). To examine the effect of flavonoids on the expression of MMP-1 in human dermal fibroblasts, cultured fibroblasts were exposed to UVA (6.3 J/cm^2) using UV light, and the MMP-1 protein and mRNA levels measured using ELISA and semi-quantitative RT-PCR, respectively. The order of MMP-1 expression inhibition appeared to be as follows: luteolin (52.20%) > myricetin (38.45%) > chrysin (32.20%) > apigenin (30.45%) > quercetin (21.81%) > kaempferol (15.45%) (Fig. 3). To further ascertain whether flavonoids regulate the expression of MMP-1 at the mRNA level, the levels of MMP-1 mRNA were examined using RT-PCR. As expected, the flavonoids markedly inhibited UVA induced the MMP-1 mRNA expression com-

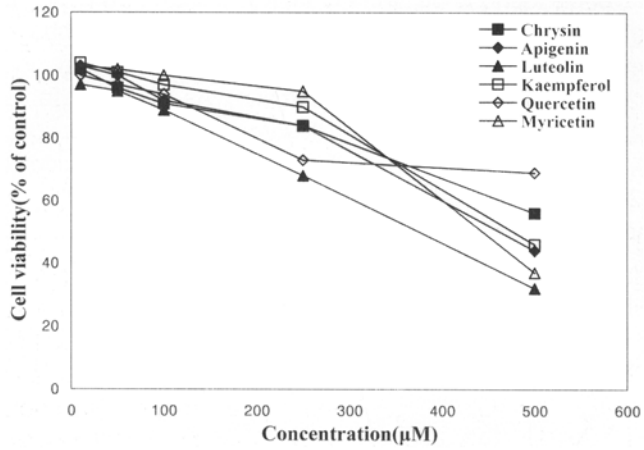


Fig. 2. The relative cell viabilities of the flavonoids on human dermal fibroblasts using the MTT assay. The cells were treated with various concentrations of flavonoids for 24 h. The results were expressed as the average plus the SD of triplicate samples.

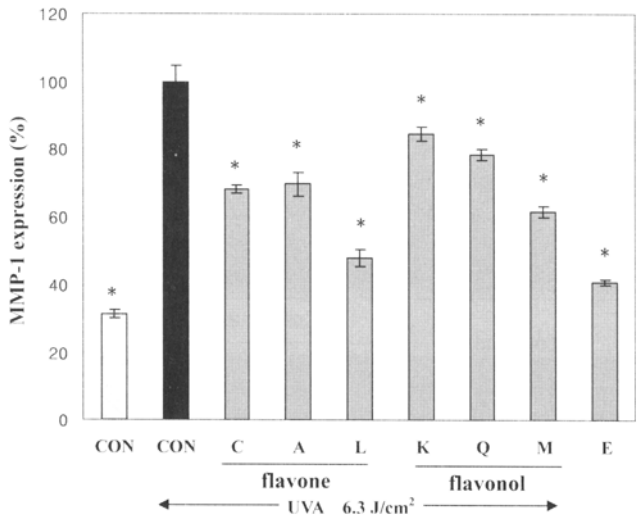


Fig. 3. Inhibitory effects of flavonoids on the expression of MMP-1 in UVA irradiated human dermal fibroblasts. The cells were cultured in the presence of flavonoids (10 mM) for 24 h. ELISA analysis was performed. The results were expressed as the average plus the SD of triple determination. *p<0.05 compared with the control. C: chrysin, A: apigenin, L: luteolin, K: kaempferol, Q: quercetin, M: myricetin., E: epigallocatechin gallate.

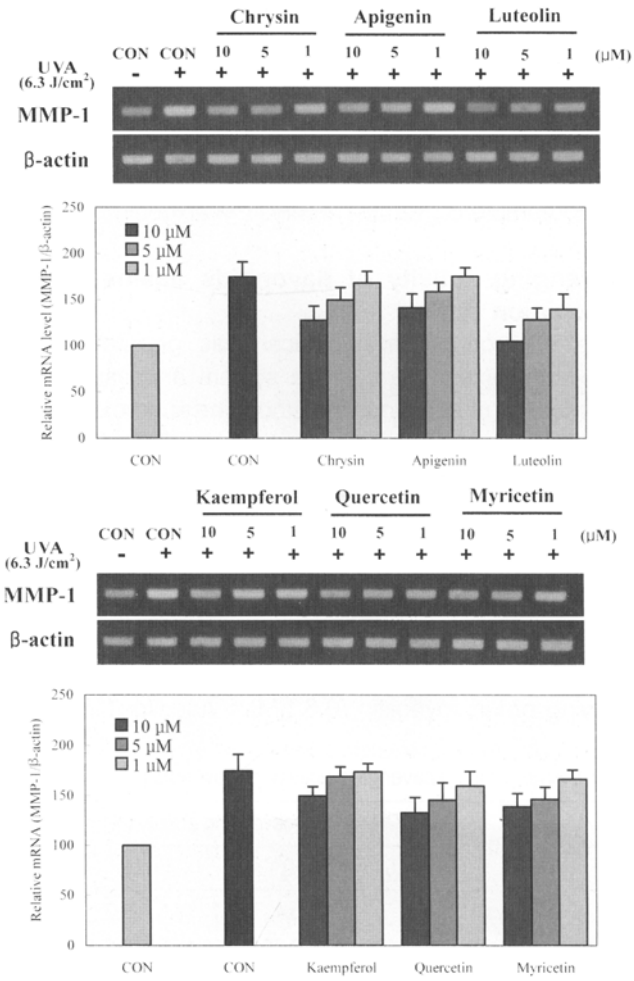


Fig. 4. Effects of flavonoids on the expression of MMP-1 mRNA in human dermal fibroblasts irradiated with UVA. The amounts of MMP-1 mRNA were analyzed 24 h post-treatment using semiquantitative RT-PCR following treatment with several doses of chrysin, apigenin, luteolin, kaempferol, quercetin, myricetin and epigallocatechin gallate. MMP-1 mRNA was normalized vs. that of the corresponding â-actin mRNA.

pared to that of the control. The MMP-1 mRNA expression was inhibited in a dose dependent manner, which with the highest dose appeared to be in the following order; luteolin (40.25%) > chrysin (27.14%) > quercetin (24.07%) > myricetin (20.58%) > apigenin (19.21%) > kaempferol (14.48%) (Fig. 4). These results did not perfectly match the inhibitory effects of flavonoids on the collagenase activity *in vitro*, but the inhibitory effect of flavonoids on the expression of MMP-1 was generally correlated with the number of hydroxyl groups in the compounds.

DISCUSSION

Repeated exposure of human skin to sunlight is considered a major factor in the premature aging of skin (photoaging). The effects of UV from sunlight on skin photoaging have been widely studied, and it has been well established that UV irradiation of cultured primary human skin fibroblasts, either *in vitro* or on human skin *in vivo* induces the expression of MMPs, including MMP-1, which play an important role in the degradation of ECM components during photoaging (Gilchrest, 1989). UV and ROS can activate the protein kinase cascades to stimulate MMP gene expression (Fisher *et al.*, 1996; Scharffetter *et al.*, 1991). It is suspected that ROS are also capable of enhancing MMP activities (Brenneisen *et al.*, 2002). In the present study, our focus was on the structure activity relationship of flavonoid compounds toward the anti-oxidant property and MMP activity in UVA irradiated human dermal fibroblasts. Our results demonstrated that flavonoids reduce the expression of MMP-1 activity and MMP-1 at both the mRNA and protein levels, as well as reduce free radical generation. The inhibitory effect of UVA-induced MMP-1 activity, expression and anti-oxidant property in human dermal fibroblasts get stronger as the numbers of -OH groups on the B-ring of flavonoid compounds are increased.

The antioxidant functions of flavonoids as scavengers of free radical are able to rapid donate a hydrogen atom to the radicals. As reviewed by Pietta (Pietta, 2000), numerous authors have investigated the antioxidant activity of flavonoids, and many attempts have been made to establish the relationship between the flavonoid structure and their radical-scavenging activity. In general, the radical-scavenging activity of flavonoids depends on their molecular structure and the substitution pattern of hydroxyl groups, i.e., on the availability of phenolic hydrogens and the possibility of stabilization of the resulting phenoxyl radicals *via* hydrogen bonding or expansion of electron delocalization (Bors *et al.*, 1990; Rice-Evans *et al.*, 1996). Previous structure-activity relationship studies on flavonoids have indicated the importance of the number and location of phenolic OH groups in the antiradical efficacy (Rice-Evans *et al.*, 1996; Van Acker *et al.*, 1996; Chen *et al.*, 1996; Cao *et al.*, 1997; Arora *et al.*, 1998, Yokozawa *et al.*, 1998; Lien *et al.*, 1999). The structural requirement considered essential for effective radical scavenging by flavonoids is the presence of 3', 4'-dihydroxy groups, i.e., an o-dihydroxy structure (catechol structure) of the B ring, which possesses electron donating properties and is a radical target. Also, the 3-OH moiety of the C ring is also beneficial for the antioxidant activity of flavonoids (Van Acker et al, 1996B). The C2-C3 double bond conjugated with a 4-keto group, which is responsible for electron delocalization from the B ring, further enhances the radical-scavenging capacity, with saturation of the 2, 3-double bond believed to cause a loss of potential activity (Rice-Evans *et al.*, 1996). The presence of both 3-OH and 5-OH groups, in combination with a 4-carbonyl function and C2-C3 double bond, increases the radical scavenging activity (Heijnen *et al.*, 2001). In the absence of an o-dihydroxy structure of the B ring, hydroxyl substituents in a catechol structure on the A-ring were able to compensate and become a larger determinant of the flavonoid antiradical activity (Arora *et al.*, 1998). Flavones possess the same basic structure as flavonoids. Some flavones tested in this study showed low free radical scavengering activities, such as apigenin and chrysin, but luteolin exhibited higher activity. Flavonols that contain more hydroxyl groups (one to six OH groups) have higher free radical and superoxide anion radical scavenging abilities, such as kaempferol, quercetin, myricetin and quercetin. Flavonols not only have a 3-hydroxyl group on the C-ring and a 3', 4'-dihydroxy structure (catechol structure) of the B-ring, but also possess the 2, 3-double bond in conjugation with a 4-oxo function on the C-ring, which are essential structural elements in the potent radical scavenging activities of flavonols. Compared with flavonol, the order of free radical scavenging activities observed in this study was as follows: myricetin (six OH) > quercetin (five OH) > kaempferol (four OH), suggesting that an increase in the number of OH groups may be important for the scavenging of free radicals.

MMPs (e.g. collagenases, gelatinases, and stromelysins), which digest collagen, gelatin (denatured collagen) and other components of the ECM, are important in both normal aging development and carcinogenesis. There are a number of different MMPs specific for various ECM components. For instance, gelatinase A (MMP-2, 72 kDa) is primarily responsible for the degradation of the helical domains of type IV collagen, the principal collagen of basement membranes, while interstitial collagenase (MMP-1) is more selective for type I collagen. A specific enzyme, vertebrate collagenase (EC 3.4.24.7), is required for the initiation of collagen degradation. This enzyme plays an essential role in the maintenance of the extracellular matrix during tissue development and remodeling. Recent

studies have demonstrated that certain MMPs are highly induced in inflamed skin, as well as in photoaged skin, and breakdown dermal matrix proteins, such as collagen and elastin; this possibly leads to prolonged skin damage and wrinkle formation (Fisher and Voorhees, 1998). Some flavonoids, such as quercetin, kaempherol and hyperoside, inhibit neutrophil elastase (MMP-12) in the micro molar concentration range (Melzing *et al.*, 2001). When different types of flavonols and catechins were examined, the flavonoids possessing polyhydroxyl groups, i.e. delphinidine, morin, myricetin and taxifolin, and the catechins with a galoyl moiety showed inhibitory activity against MMP-2, MMP-9 and MMP-12 (Demeule *et al.*, 2000; Sartor *et al.*, 2002). However; to date, the direct modulatory effect of flavonoids on collagenase or MMP-1 (mammalian collagenase-1) has rarely been demonstrated, despite the importance of MMP-1 and collagen breakdown in inflammatory skin diseases and photoaing. Only epicatechin gallate and epigallocatechin gallate, isolated from tea, have been described to inhibit collagenase at high concentrations (Makimura *et al.*, 1993). Therefore, the collagenase inhibitory effect of flavonoids was investigated, and found that flavonols were stronger inhibitors than flavones, implying that the C-3-hydroxyl group is important in the higher inhibitory activity (apigenin vs. kaempferol and luteolin vs. quercetin). Particularly, kaempferol, quercetin and myricetin showed considerable inhibition.

The mitogen-activated protein kinase (MAPK) signal transduction pathways play important roles in regulating a variety of cellular functions, including MMP expression (Robinson and Cobb, 1997). Fisher *et al.* suggested that UV radiation activates growth factor receptors, which induce the activation of protein kinase cascades, such as the MAPK cascade (Fisher and Voorhees, 1998). This activation results in an increase in the expressions of c-Jun and c-Fos, which form the AP-1 complex. Transcriptions of several MMPs, including MMP-1, -3, and –9, are regulated by AP-1 (Huang *et al.*, 1997). The downstream effectors of the MAPK include several transcription factors, including Elk-1, c-Fos and c-Jun. Moreover, the elevated AP-1 activity is responsible for the degradation of ECM proteins, such as collagen; by inducing MMPs. ROS generation plays a critical role in the MAPK-mediated signal transduction triggered by UV (Whisler *et al.*, 1995). In addition, ROS increase the expression of MMP-1 in human skin fibroblasts; whereas, ROS scavengers inhibit UV-induced AP-1 activation and the expression of MMP-1 (Wenk *et al.*, 1999). Theses results demonstrate that flavonoids reduce the expression of MMP-1 at both the mRNA and protein levels. In the case of flavonols, the inhibitory effect of UV induced MMP-1 expression in human dermal fibroblasts becomes stronger with increases in the number of -OH groups on the B-ring. Conversely, with flavones, the inhibitory effect of UV induced MMP-1 expression in human dermal fibroblasts does not correlate with the number of -OH groups on the B-ring.

In conclusion, this study has demonstrated that the effects of antioxidative activity and inhibition on the collagenase activity and expression of MMP-1 caused by UVA in human dermal fibroblasts depends on the number and order of OH groups in the flavonoids structure. Also, it is suggested that flavonoids may also prevent the progression of skin photoaging.

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REFERENCES

Arora, A., Nair, M.G., and Strasburg, G. M., Structure–activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radic. Biol. Med.*, 24, 1355-1363 (1998).

Blois, M. S., Antioxidant determinations by the use of a stable free radical. *Nature*, 181, 1199-1200 (1958).

Bors, W., Heller, W., Michel, C., and Saran, M.,. Flavonoids as antioxidants: determination of radical scavenging efficiencies. *Methods Enzymol.*, 186, 343-355 (1990).

Brenneisen, P., Sies, H., and Scharffetter-Kochanek, K., UV-B irradiation and matrix metalloproteinases: from induction via signaling to initial events. *Ann. N. Y. Acad. Sci.,* 973, 31-43 (2002).

Cao, G., Sofic, E., and Prior, R. L., Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radi. Biol. Med.,* 22, 749-760 (1997).

Chambers, A. F. and Matrisian, L. M., Changing views of the role of matrix metalloproteinases in metastasis. *J. Natl. Cancer Inst.*, 89, 1260-1270 (1997).

Chen, Z. Y., Chan, P. T., Ho, K. Y., Fung, K.,P., and Wang, J., Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. *Chem. Phys. Lipids*, 79, 157-163 (1996).

Cos, P., Ying, L. Y., Calomme, N., Hu, J. H., Cimanga, K., Van Poel, B., Pieters, L., Vlietinck, A. J., and Berghe, D. V., Structure activity relationships and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J. Nat. Prod.*, 61, 71-76 (1998).

Cotelle, N., Bernier, J. L., Catteau, J. P., Pommery, J., Wallet, J. C., and Caydou, E. M., Antioxidant properties of hydroxyl-flavones. *Free Radi. Biol. Med.*, 20, 35-43 (1996).

Cunningham, M. L., Krinsky, N. I., Giovanazzi, S. M., and Peak, M. J., Superoxide anion is generated from cellular

metabolites by solar radiation and its components. *Free Radi. Biol. Med.*, 1, 381-385 (1985).

Demeule, M., Brossard, M., Page, M., Gingras, D., and Beliveau, R., Matrix metalloproteinase inhibition by green tea catechins. *Biochim. Biophys. Acta* 1478, 51-60 (2000).

Ende, C. and Gebhardt, R., Inhibition of matrix metallproteinase-2 and -9 activity by selected flavonoids. *Planta Med.*, 70, 1006-1008 (2004).

Fisher, G. J., Datta, S. C., Talwar, H. S., Wang, Z. Q., Varani, J., Kang, S., and Vorhees, J. J., Molecular basis of sun-induced premature skin aging and retinoid antagonism. *Nature*, 379, 335-339 (1996).

Fisher, G. J. and Voorhees, J. J., Molecular mechanism of photoaging and its prevention by retinoic acid: ultraviolet irradiation induces MAP kinase signaling transduction cascade that induce AP-1 regulated matrix metalloproteinases that degrade human skin *in vivo*. *J. Invest. Dermatol. Symposium Proceedings*, 3, 61-68 (1998).

Fisher, G. J, Wang, Z. Q,, Datta, S. C., Varani, J., Kang, S., and Voorhees, J. J., Pathophysiology of premature skin aging induced by ultraviolet light. *N. Engl. J. Med.*, 337, 1419-1428 (1997).

Furuno, K., Akasako, T., and Sugihara, N., The contribution of the pyrogallol moiety to the superoxide radical scavenging activity of flavonoids. *Biol. Pharm. Bull.*, 25, 19-23 (2002).

Gamet-Payrastre, L., Manenti, S., Gratacap, M. P., Tulliez, J., Chap, H., and Payrastre, B., Flavonoids and the inhibition of PCK and PI 3-kinase. *Gen. Pharmacol.*, 32, 279-286 (1999).

Gilchrest, B.A., Skin aging and photoaging: an overview. *J. Am. Acad. Dermatol.*, 21, 610-613 (1989).

Hanson, K. M., and Clegg, R. M., Observation and quantification of ultraviolet-induced reactive oxygen species in ex vivo human skin. *Photochem. Photobiol.*, 76, 57-63 (2002).

Heijnen, C. G. M., Haenen, G. R. M. M., Van Acker, F. A. A., Van der Vijgh, W. J. F., and Bast, A., Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. *Toxicol. In Vitro* 5, 3-6 (2001).

Huang, C., Schmid, P. C., Ma, W. Y., Schmid, H. H., and Dong, Z., Phophatidylinositol-3 kinase is necessary for 12-O-tetradecanoylphorbol-13-acetate-induced cell transformation and activated protein 1 activation. *J. Biol. Chem.*, 272, 4187-4194 (1997).

Jenkins, G., Molecular mechanisms of skin aging. *Mech. Aging Dev.*, 123, 801-810 (2002).

Kligman, A. M., Early destructive effects of sunlight on human skin. *J. Am. Med. Assoc.*, 210, 2377-2380 (1969).

Lien, E. J., Ren, S., Bui, H. H., and Wang, R., Quantitative structure-activity relationship analysis of phenolic antioxidants. *Free Radi. Biol. Med.*, 26, 285-294 (1999).

Makimura, M., Hirasawa, M., Kobayashi, K., Indo, J., Sakanaka, S., Taguchi, T., and Otake, S., Inhibitory effect of tea catechins on collagenase activity. *J. Periodontol.*, 64, 630-636 (1993).

Melzig, M. F., Loser, B., and Ciesielski, S., Inhibition of neutrophil elastase activity by phenolic compounds from plants. *Pharmazie*, 56, 967-970 (2001).

Middleton, Jr, E., Kandaswami, C., and Theoharides, T. C., The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.*, 52, 673-751 (2000).

Nagase, H., Sasaki, K., Kito, H., Haga, A., and Sato, T., Inhibitory effect of delphinidin from Solanum melonena on human fibrosarcoma HT-1080 invasiveness *in vitro*. *Planta Med.*, 64, 216-219 (1998).

Ozcelik, B., Lee, J. H., and Min, D. B., Effects of light, oxygen and pH on the 2,2-diphenyl-l-picrylhydrazyl (DPPH) method to evaluate antioxidants. *J. Food Sci.*, 68, 487-490 (2003).

Parejo, I., Viladomat, F., Bastida, J., Rosas-Romero, A., Flerlage, N., Burillo, J., and Codina, C., Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled Mediterranean herbs and aromatic plants. *J. Agric. Food Chem.*, 50, 6882-6890 (2002).

Pietta, P. G., Flavonoids as antioxidants. *J. Nat. Prod.*, 63, 1035-1042 (2000).

Rice-Evans, C. A., Miller, N. J., and Paganga, G., Structure-antioxidant activity relationship of flavonoids and phenolic acids. *Free Radi. Biol. Med.*, 20, 933-956 (1996).

Robinson, M. J. and Cobb, M. H., Mitogen-activated protein kinase pathways. *Curr.t Opin. Cell Biol.*, 9, 180-186 (1997).

Sartor, L., Pexxato, E., Dell'Aica, I., Caniato, R., Biggin, S., and Garbisa, S., Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatroy and anti-invasion drug design. *Biochem. Pharmacol.*, 64, 229-237 (2002).

Sato, T., Koike, L., Miyata, Y., Hirata, M., Mimaki, Y., Sashida, Y., Yano, M., and Ito, A., Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and -9 in human fibrosarcoma HT-1080 cells. *Cancer Res.*, 62, 1025-1029 (2002).

Scharffetter-Kochanek, K., Brenneisen, P., and Wenk, J., Photoaging of the skin from phenotype to mechanisms. *Exp. Gerontol.* 35, 307-316 (2000).

Scharffetter, K., Wiaschek, M., Hogg, A., Bolsen, K., Schothorst, A., Goerz, G., Krieg, T., and Plewig, G., UVA irradiation induces collagenase in human dermal fibroblasts in vitro and *in vivo*. *Arch. Dermatol. Res.*, 283, 506-511 (1991).

Shang, J., Schwarz, C., Sanchez Ruderisch, H., Hertting, T., Orfanos, C. E., and Tebbe, B., Effects of UVA and L-ascorbic acid on nuclear factor-kappa B in melanocytes and in HaCaT keratinocytes. *Skin Pharmacol. Appl. Skin Physiol.*, 15, 353-359 (2002).

Van Acker, S. A. B. E., Van den Berg, D. J., Tromp, M. N. J. L., Griffioen, D. H., Van Bennekom, W. P., Van der Vijgh, W. J. F., and Bast, A., Structural aspects of antioxidant activity of flavonoids. *Free Radi. Biol. Med.*, 20, 331-342 (1996).

Van Acker, S. A. B. E., De Groot, M. J., Van den Berg, D. J., Tromp, M. N. J. L., Den Kelder, G. D. O., Van der Vijgh, W. J. F., Bast, A., A Quantum chemical explanation of the antioxidant activity of flavonoids. *Chem. Res. Toxicol.*, 9, 1305-1312 (1996B).

Varani, J., Warner, R. L., Gharaee-Kermani, M., Phan, S. H., Kang, S., Chung, J. H., Wang, Z. Q., Datta, S. C., Fisher, G. J., and Voorhees, J. J., Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metallproteinases and stimulates collagen accumulation in naturally aged human skin. *J. Invest. Dermatol.*, 114, 480-486 (2000).

Vile, G. F. and Tyrrell, R. M., UVA radiation-induced oxidative damage to lipids and proteins *in vitro* and in human skin fibroblasts is dependent on iron and singlet oxygen. *J. Invest. Dermatol.*, 18, 721-730 (1995).

Wenk, J., Brenneisen, P., Wlaschek, M., Poswig, A., Briviba, K., Oberley, T. D., and Scharffetter-Kochanek, K., Stable overexpression of manganese superoxide dismutase in mitochondria identifies hydrogen peroxide as a major oxidant in the AP-1-mediated induction of matrix-degrading metalloproteinase-1. *J. Biol. Chem.*, 274, 25869-25876 (1999).

Went, J., Brenneisen, P., and Meewes, C., UV-induced oxidative stress and photoaging. *Curr. Probl. Dermatol.*, 29, 74-82 (2001).

Whisler, R. L., Goyette, M. A., Grants, I. S., and Newhouse, Y. G., Sublethal levels of oxidant stress stimulate multiple serine/threonine kinases and suppress protein phosphatases in Jurkat T cells. *Arch. Biochem. Biophys.*, 319, 23-35 (1995).

Yokozawa, T., Chen, C. P., Dong, E., Tanaka, T., Nonaka, G. I., and Nishioka, I., Study on the Inhibitory Effect of Tannins and Flavonoids against the 1,1-Diphenyl-2-picrylhydrazyl Radical. *Biochem. Pharmacol.*, 56, 213-222 (1998).