Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : December 21, 2022
Accepted : January 16, 2023
Published online : March 31, 2023
doi:10.24659/gsr.10.1_6

Original article

Antiglycative effect of plant extract complex

Masayuki Yagi [1)], Chieko Sakiyama [1)], Kyoko Mori [2)], Masato Ohta [2)], Kiyoshi Amami [2)], Yoshikazu Yonei [1)]

1) Anti-Aging Medical Research Center and Glycative Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan
2) Cocochicosme Co., Ltd., Tokyo, Japan

Abstract

Accumulation of advanced glycation end products (AGEs) in skin tissues due to glycative stress is one of the factors that accelerate skin aging, including decreased elasticity and loss of texture. Suppression of glycative stress is called anti-glycation or glycation care. For anti-glycation of the skin, there are treatments from the inside of the body such as diet and approaches from the outside of the body such as skin care preparations. Furthermore, anti-glycation includes the suppression of postprandial hyperglycemia, suppression of glycation reactions, and decomposition and excretion of AGEs. Various plant materials have been shown to have anti-glycation effects. However, the glycation reaction in the body that leads to the formation of AGEs is a complex multi-pathway reaction, and it is thought that a multi-component approach may be useful. This study was conducted to evaluate the anti-glycation effects of five plant extract complexes (sea buckthorn fruit, Chinese blackberry tea, tea plant leaves, loquat leaves, and rosemary leaves) that can be used in skin care formulations. To evaluate the anti-glycation effects of plant extract complex (PEC) on skin, we examined its inhibitory effects on glycation reaction, AGE cross-link cleavage, inhibitory formation of glycated protein cross-link, glycated protein cross-link cleavage, and antioxidant activity. As results, PEC inhibited the production of fluorescent AGEs in protein glycation models of human serum albumin (HSA), collagen, and keratin. PEC also inhibited the production of pentosidine, N^{ε}-(carboxymethyl)lysine (CML), 3-deoxyglucosone (3DG), and glyoxal (GO) in a keratin-glucose glycation model. Furthermore, PEC has AGE cross-link-cleavage effect, inhibits protein cross-link formation in a lysozyme-glucose glycation cross-linking model, and degrades proteins dimerized by glycation. PECs may be useful in preventing glycation of skin by suppressing AGE accumulation in skin proteins and preventing functional deterioration caused by the formation of glycated protein cross-links.

***KEY WORDS*:** inhibition of glycation reaction, protein cross-linking degradation, plant extract complex, skin aging

Introduction

Accumulation of advanced glycation end products (AGEs) in skin tissues due to glycative stress is one of the factors that contribute to skin aging, including loss of elasticity and texture. In addition, oxidation is a factor that promotes glycative stress [1)]. Suppression of glycative stress is called anti-glycation or glycation care. Anti-glycation for skin includes treatments from the inside of the body, such as dietary intake, and treatments s from the outside, such as application of skin care products.

Anti-glycation includes suppression of postprandial hyperglycemia, suppression of glycation reaction, and degradation and excretion of AGEs [2)]. Postprandial hyperglycemia can be suppressed by taking glycolytic enzyme inhibitors with meals or by improving dietary methods [3)]. Aminoguanidine (AG) is a compound known to inhibit glycation reaction [4, 5)]. However, AG cannot be added in foods and cosmetics due to side effects such as decreased liver function and vitamin B6 deficiency. On the other hand, natural

Contact Address: Professor Masayuki Yagi, PhD
Anti-Aging Medical Research Center / Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University
1-3, Tatara Miyakodani, Kyotanabe-shi, Kyoto, 610-0394 Japan
TEL&FAX: +81-774-65-6394 e-mail: myagi@mail.doshisha.ac.jp
Co-authors; Sakiyama C, csakiyam@mail.doshisha.ac.jp;
Ohta M, masato.ohta@cocochicosme.com; Mori K, kyoko.mori@cocochicosme.com;
Amami K, yj@cocochi.cn; Yonei Y, yyonei@mail.doshisha.ac.jp

products including many plant materials such as tea [6], vegetables and herbs [7], and fruits [8] have been reported to inhibit glycation reactions.

N-phenacylthiazolium bromide (PTB) is known as a substance that cleaves AGE cross-links by glycation [9]. PTB cleaves α-diketones, a type of cross-linked structure of glycated proteins [10]. Natural products with similar effects to PTB include extracts of such as pomegranate (*Punica granatum*) [11], water chestnuts (*Trapa bicornis*) [12], fennel (*Trigonella foenum-graecum*), fenugreek (*Foeniculum vulgare*), hibiscus (*Hibiscus sabdariffa*) [13]. These AGE cross-link cleavage agents may be involved in the degradation and excretion of AGEs. In addition, edible purple chrysanthemum (*Chrysanthemum morifolium*) extract has been shown to inhibit the formation of glycated protein cross-links and cleave glycated protein cross-links as well as AGE cross-link cleaving action [14]. Plants with glycative stress inhibitory effects include flavonoids [15] and tannins [16]. These components have antioxidant properties.

Glycation reactions leading to the formation of AGEs *in vivo* are complex and multi-pathway [17]. For this reason, it is considered useful to suppress the formation of AGEs, which have different formation pathways, by using multiple components [18]. *In vitro* studies of four herbal mixtures showed that they inhibited the formation of various AGEs [19]. In addition, intake studies of herbal mixtures showed that they inhibited the accumulation of AGEs in blood and skin [20-22] and improved skin aging indices [23]. A skin care formulation containing mugwort (*Artemisia indica*) extract, which has AGE cross-link-cleaving activity, has been shown to improve skin elasticity in clinical studies [24, 25]. Thus, materials that inhibit AGE formation and degrade AGE crosslink structures in *in vitro* anti-glycation studies have the potential to reduce the effects of glycative stress in humans as foods or skin care cosmetics.

In this study, we evaluated the anti-glycation effects of five plant extract complexes (PECs) that can be used in skin care formulations by examining their inhibitory effects on glycation reactions, AGE cross-link cleavage, glycated protein cross-link formation, glycated protein cross-link cleavage, and antioxidant effects.

Materials and methods

1) Reagents

The reagents used were purchased from the following manufacturers and used. Human serum albumin (HSA, lyophilized powder, ≥ 96%, agarose gel electrophoresis), methylglyoxal solution (40% in H_2O; MGO), *N*-phenacylthiazolium bromide (PTB) was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Collagen Type I (bovine skin, Pepsin degradation) is supplied by Nippi (Tokyo, Japan). Wool-derived keratin (keratin, partially sulfonated, 5% in water) is supplied by Tokyo Chemical Industry (Tokyo, Japan). Aminoguanidine hydrochloride (AG), glyoxal (40% glyoxal solution; GO), 2,3-diaminonaphthalene (DAN), 1-phenyl-1,2 (±)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Fujifilm Wako Pure Chemical Industries (Osaka, Japan), 3-deoxyglucosone (3DG) from Dojin Chemical Laboratory (Kumamoto, Japan), CircuLex CML/Nε-(carboxymethyl) lysine) ELISA Kit is manufactured by MBL (Aichi, Japan). Pentosidine (pentosidine-TFA salt) was from Peptide Institute (Osaka, Japan). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was from Cayman Chemical (Ann Arbor, MI, USA). Other reagents were purchased from Fujifilm Wako Pure Chemical Industries or Nakalai Tesque (Kyoto, Japan) in special or HPLC grade.

2) Samples

The plant extract complex (PEC) was made by mixing 1,3 butylene glycol (BG) extracts of sea buckthorn (*Hippophae rhamnoides*) fruit, Chinese blackberry (*Rubus suavissimus*) tea, tea plants (*Camellia sinensis*) leaves, loquat (*Eriobotrya japonica*) leaves, and rosemary (*Salvia rosmarinus*) leaves. The BG extract of mugwort leaves was compared as a working control solution (RF) for known anti-glycation components [24, 25]. PEC and RF were diluted with BG solution (dissolving solution) as appropriate and used as sample solutions. The sample concentration was 100% of the extracted solution concentration of each sample stock solution. These samples were provided by Cosmo Beauty (Osaka, Japan).

3) Protein-glucose glycation model

A protein-glucose glycation reaction model was used to verify the inhibitory effect of the glycation reaction, based on a previous report [26]. The composition of the reaction solution was prepared by adding 1/10 of the sample solution to 0.1 mol/L phosphate buffer (pH 7.4) containing protein and glucose. The protein and glucose concentrations were HSA 8 mg/mL and glucose 0.2 mol/L, collagen 1.2 mg/mL and glucose 0.4 mol/L, and keratin 5 mg/mL and glucose 0.2 mol/L. For the glycation reaction, phosphate buffer, protein solution, glucose solution, and sample solution were all added (solution A), purified water was added in place of the glucose solution in A (solution B), dissolving solution was added in place of the sample solution in A (solution C), and purified water was added in place of the glucose solution in C (solution D) were prepared and incubated at 60 °C. Incubation time was 40 hours for HSA and 10 days for collagen and keratin.

4) Measurement of AGEs

Fluorescent AGEs (F-AGEs) were measured, according to a previous report [26], AGE-derived fluorescence (excitation wavelength 370 nm / fluorescence wavelength 440 nm) in 200 μL of the glycation reaction solution after incubation by using a black microplate. Pentosidine was measured by HPLC after hydrolysis of 50 μL of the glycation reaction mixture with 6 N HCl at 110 °C for 18 h according to a previous report [27]. CML was measured using the CircuLex CML/Nε-(Carboxymethyl)lysine ELISA Kit after appropriate dilution of the reaction mixture.

5) Measurement of glycation reaction intermediates

The glycation reaction intermediates were measured for 3DG, GO, and MGO according to previous reports [28, 29]. After

deproteinizing 100 μL of the glycation reaction solution with perchloric acid, DAN was added under alkaline conditions for labeling, followed by HPLC measurement.

6) Evaluation of the inhibitory effect on glycation reaction

As an evaluation of the inhibitory effect on glycation reaction, the inhibition rate (%) of production of AGEs and glycation reaction intermediates was calculated by the following equation according to a previous report [26],. The positive control for inhibition of glycation reaction was AG.

$$\text{Inhibition (\%)} = \{1 - (A - B) / (C - D)\} \times 100$$

The half maximal inhibitory concentration (IC_{50}; mg/mL) was calculated from the inhibition rates of the three concentrations for each sample [26,30]. A smaller IC_{50} value indicates a stronger inhibitory effect on the glycation reaction.

7) Evaluation of cleavage of AGE cross-linkings

The cleavage of AGE cross-link was measured according to a previous report [31-33]. PPD was used as a model substance for AGE cross-linking, and the cleavage rate of α-diketone of PPD by the sample was measured. The mixture solution of sample solution, PPD, and phosphate buffer (pH 7.4) was reacted at 37 °C for 8 hours, and after the reaction was stopped by adding hydrochloric acid. The α-diketone of PPD was cleaved by the sample, and the amount of free benzoic acid was measured by HPLC under the same conditions as previously reported [32]. The AGE cross-link cleavage rate was calculated using the equation shown below, based on the principle that "when the α-diketone of a PPD molecule is cleaved, 1 mol of PPD yields 1 mol of benzoic acid".

$$\text{AGE cross-link cleavage rate (\%)} = \{(A - B - R) / C\} \times 100$$

A; Amount of benzoic acid in the reaction solution,
B; Amount of benzoic acid in the sample,
R; Amount of benzoic acid in the reaction solution when only dissolving solution was added,
C; amount of PPD used in the reaction

8) Inhibitory actions on glycation-induced protein cross-linking

The glycation-induced lysozyme-glucose cross-linking model was used for evaluation of the inhibitory actions of glycation-induced protein cross-link formation according to a previous report [14, 34, 35]. The reaction solution was prepared by adding the sample solution to 0.1 mol/L phosphate buffer (pH 7.4) containing lysozyme and glucose. Reaction solutions were prepared by adding all of the phosphate buffer, lysozyme solution, glucose solution, and sample solution (solution A), solution with purified water added in place of the glucose solution in A (solution B), solution with dissolving solution added in place of the sample solution in A (solution C), solution with purified water added in place of the glucose solution in C (solution D) were prepared and incubated at 60 °C for 40 hours.

After completion of the reaction, the reaction solution was centrifuged and the supernatant was subjected to 4-20 % SDS-PAGE. The gel was stained with CBB Stain One (Nakalai Tesque, Kyoto, Japan), the electrophoretic gel images were imaged, and the intensities of the lysozyme dimer (28.6 kDa) and trimer (42.9 kDa) bands were analyzed with Image J. AG was used as a positive control substance for inhibition of glycated protein cross-link formation. The inhibition rate of glycated protein cross-link formation was calculated based on the following formula.

$$\text{Percentage inhibition of glycated protein cross-link formation (\%)} = \{1 - (A - B) / (C - D)\} \times 100$$

A-D; band intensity of lysozyme dimer or trimer in SDS-PAGE gel stained images of each reaction solution

9) Evaluation of cleavage of glycated-protein cross-link

The cleavage of glycated-protein cross-link (CGPC) was measured by referring to previous reports [14, 33], glycated lysozyme was prepared, and the degradation rate of lysozyme dimer by the sample was measured. Glycated lysozyme was prepared by reacting lysozyme with 0.1 mol/L phosphate buffer (pH 7.4) containing glucose at 60 °C for 40 hours, followed by ultrafiltration to remove low molecular weight substances. Reaction solutions were prepared by adding sample solution in 0.05 mol/L phosphate buffer containing glycated lysozyme (solution S), and dissolving solution in place of sample solution in S (solution R), and incubated at 37 °C for 16 hours to react. The reaction solution was then centrifuged and the supernatant was subjected to 4-20 % SDS-PAGE. After gel electrophoresis, gels were stained with CBB Stain One and imaged with a scanner GT- X830 (Epson, Suwa, Nagano, Japan), and the intensity of lysozyme monomer (M) and dimer (D) bands were analyzed with Image J. PTB was used as a positive control substance for CGPC. The glycated protein cross-link cleavage rate was calculated based on the following equation.

$$\text{Glycated-protein cross-link cleavage rate (\%)} = [1 - \{(D_S/M_S) / (D_R/M_R)\}] \times 100$$

S ; Band intensity of lysozyme in sample solution
R ; Band intensity of lysozyme in sample dissolving solution
M; Monomer band of lysozyme
D ; Dimer band of lysozyme

10) Evaluation of antioxidant activity

The DPPH radical scavenging activity corresponding to the sample solution was measured using the slope of the regression line generated by Trolox, referring to previous reports [36,37]. DPPH radical scavenging activity was measured, by adding sample extracts, MES buffer (pH 6.0), DPPH solution, and 50 % ethanol solution into the wells of a microplate, followed by a 20-minute reaction at room temperature, absorbance at 520 nm.

The slope of the linear regression line between the amount of sample extract added and absorbance was then determined. The DPPH radical scavenging activity value

was calculated as the Trolox equivalent (TE) of the sample extract by dividing the slope of the linear regression line of the absorbance, obtained from the reaction solution of Trolox (a water-soluble analog of vitamin E) that was reacted concurrently with the sample, by the slope of the regression line of the sample extract.

Statistical analysis

Measurements are expressed as mean ± standard deviation (SD). Tukey's test or t-test was used to compare measurements. Statistical analysis results were considered significant at a risk rate of less than 5%.

Results

Inhibition of fluorescent AGE formation in protein-glucose glycated reaction models

PEC and RF inhibition rate of F-AGE formation increased with sample concentration in all three reaction models using HSA, collagen, and keratin as model proteins (***Fig. 1***). The IC_{50} of PEC inhibition of F-AGE formation was lowest for collagen (0.175%), followed by HSA (0.290%) and keratin (2.92%, ***Table 1***). Similarly, the IC_{50} for RF was lowest for HSA (0.826%), followed by collagen (1.557%) and keratin (greater than 2.0%). The IC_{50} of PEC inhibition of F-AGE formation was 0.4-fold for HSA, 0.5-fold for collagen, and less than 0.4-fold for keratin compared to RF. The IC_{50} values of PEC were lower than those of RF for all model proteins.

Inhibition of AGEs and glycation intermediates formation in the keratin-glucose glycation reaction model

A keratin-glucose glycation reaction model was used to verify the inhibitory effects of PEC and RF on pentosidine, CML, 3DG, GO, and MGO formation (***Table 2***). The IC_{50} for PEC was lowest for CML and GO (both <1%), followed by MGO (1.9%), 3DG (4.7%), and pentosidine (9.6%). Similarly, IC_{50} values for RF were lowest for CML (0.9%) and highest for GO (1.2%), MGO (4.1%), and pentosidine (8.8%), in that order. IC_{50} values could not be calculated due to little inhibition rate of formation of MGO for PEC and 3DG for RF. IC_{50} values for AG could not be calculated due to small effect except for GO (0.1%).

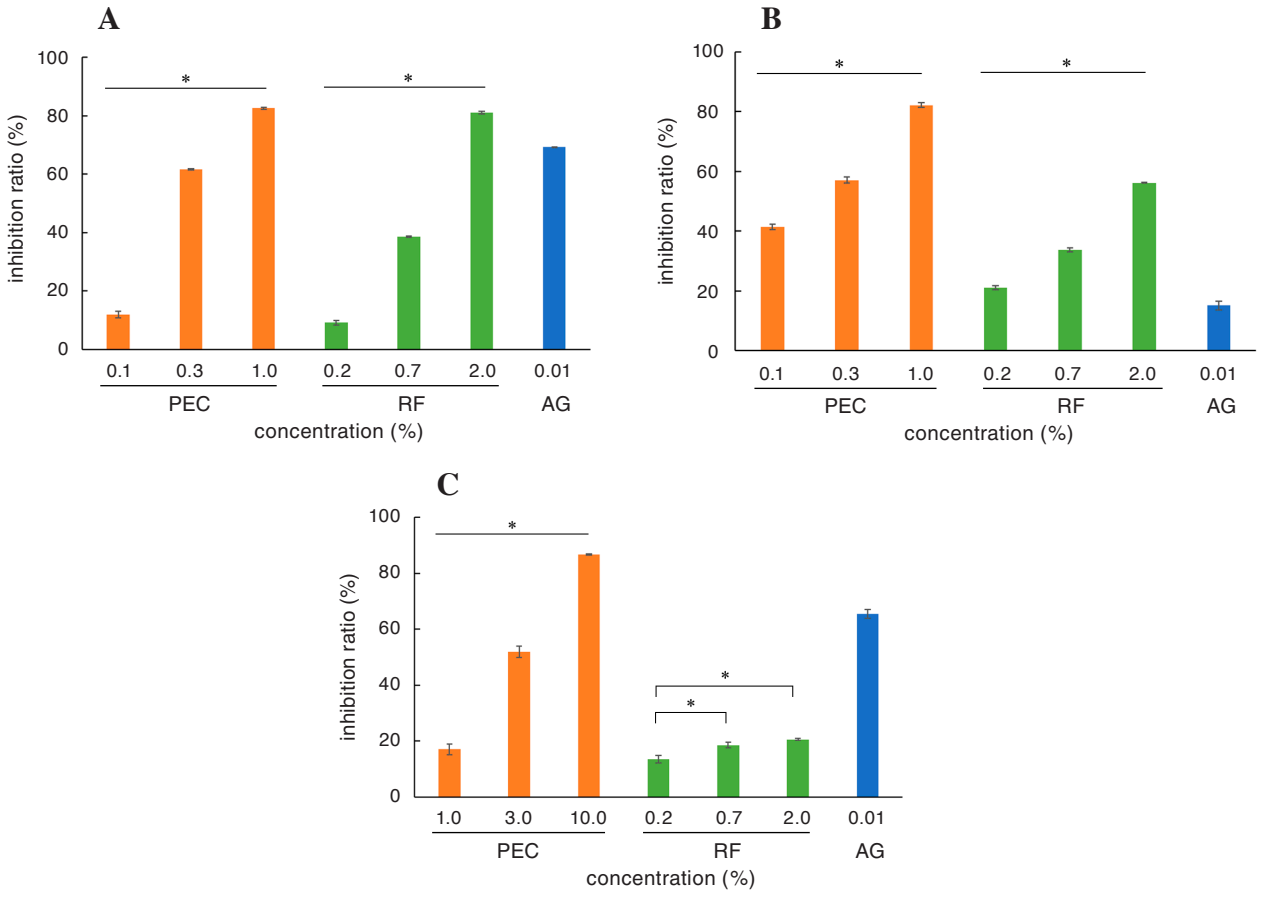

Fig. 1. **Inhibitory activity of F-AGE formation.**

A, HSA-glucose model; **B**, collagen-glucose model; **C**, keratin-glucose model. Results are expressed as mean ± standard deviation, n = 3, * $p < 0.05$ vs different concentration samples by Tukey's test; PEC, plant extract complex; RF, *Artemisia indica Willd. var. maximowiczii* leaf extract, AG, aminoguanidine; F-AGE, fluorescent advanced glycation end product; HSA, human serum albumin.

Table 1. Half maximal inhibitory concentration (IC_{50}) on the protein-glucose glycation model.

Model protein	PEC[1] (%)	RF[2] (%)	AG[3] (%)
HSA	0.290	0.826	0.006
collagen	0.175	1.557	0.049
keratin	2.92	2.0 <	0.007

1) plant extract complex, 2) *Artemisia indica Willd. var. maximowiczii* leaf extract, 3) aminoguanidine; positive control of glycation inhibitor. HSA, human serum albumin.

Table 2. Half maximal inhibitory concentration (IC_{50}) on the keratin-glucose glycation model.

AGE compound	PEC[1] (%)	RF[2] (%)	AG[3] (%)
pentosidine	9.6	8.8	NC[8]
CML[4]	1.0 >	0.9	NC
3DG[5]	4.7	NC	NC
GO[6]	1.0 >	1.2	0.1
MGO[7]	1.9	4.1	NC

1) plant extract complex, 2) *Artemisia indica Willd. var. maximowiczii* leaf extract, 3) aminoguanidine; positive control of glycation inhibitor, 4) N^{ε}-carboxymethyl lysine, 5) 3-deoxyglucosone, 6) glyoxal, 7) methylglyoxal, 8) can't calculation.

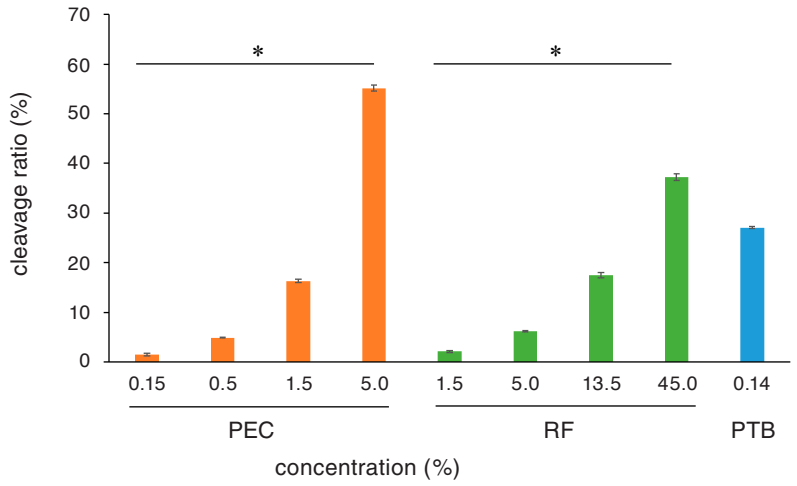

Fig. 2. **AGE cross-link cleavage activity.**

Results are expressed as mean ± standard deviation, n = 3; * $p < 0.05$ vs different concentration samples by Tukey's test; PEC, plant extract complex; AGE, advanced glycation end product; RF, *Artemisia indica Willd. var. maximowiczii* leaf extract, PTB, N-phenacylthiazolium bromide.

AGE cross-link cleavage

The AGE cross-link cleavage rates of PEC and RF increased with sample concentration. At a sample concentration of 1.5%, the AGE cross-linking cleavage rate of PEC (16.3 ± 0.4%) was 7.8-fold higher than that of RF (2.1 ± 0.2%, $p < 0.05$, ***Fig. 2***).

Inhibition of glycated protein cross-link formation

The inhibition rates of lysozyme trimer and dimer formation by PEC, RF, and AG increased with sample concentration (***Table 3***). The IC_{50} values for inhibition of glycated protein cross-link formation were lowest for AG for both the dimer and trimer, followed by PEC and RF. The inhibition rate for dimer formation at a sample concentration of 0.3% was 2.8-fold higher for PEC (54.9 ± 3.5%) than for RF (19.7 ± 4.3%, $p < 0.05$).

Cleavage of Glycated Protein Cross-link

The glycated proteins cross-link cleavage rate increased with the sample concentration. However, there was no difference in the cross-link cleavage rate of RF at sample concentrations between 0.2~2% (***Fig. 3, 4***). The cross-link cleavage rate of 0.5% PEC was 30.9 ± 7.8%, and 1.7 times higher than that of 0.5% RF (18.6 ± 3.5%, $p < 0.05$).

Table 3. Inhibitory effect of PEC, RF and aminoguanidine on cross-linking formation in the lysozyme-glucose reaction model.

sample	conc. (%)	dimer			trimer		
		inhibition ratio (%)		IC_{50} (%)	inhibition ratio (%)		IC_{50} (%)
PEC[1)]	0.1	43.3 ± 4.1	*	0.19	88.9 ± 0.8		0.1 >
	0.3	54.9 ± 3.5			89.2 ± 3.3		
	1.0	68.7 ± 2.0			86.3 ± 1.1		
RF[2)]	0.1	-1.6 ± 3.7	*	1.6	19.5 ± 2.8	*	0.24
	0.3	19.7 ± 4.3			66.8 ± 3.1		
	1.0	41.2 ± 3.7			84.6 ± 4.0		
AG[3)]	0.01	25.7 ± 6.8	*	0.053	79.5 ± 3.8	*	0.01 >
	0.03	42.4 ± 3.8			92.9 ± 1.4		
	0.1	58.9 ± 8.3			98.6 ± 1.2		

Results are expressed as mean ± standard deviation, n = 3; * $p < 0.05$ vs different concentration samples by Tukey's test; IC_{50}, half maximal inhibitory concentration. 1) plant extract complex, 2) *Artemisia indica Willd. var. maximowiczii* leaf extract, 3) aminoguanidine; positive control of glycation inhibitor. IC_{50}, half maximal inhibitory concentration.

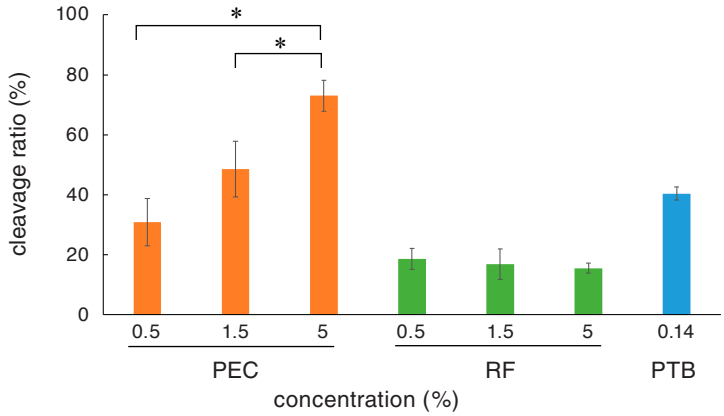

Fig. 3. **Cleavage of glycated-protein cross-link activity.**

Results are expressed as mean ± standard deviation, n = 3; * $p < 0.05$ vs different concentration samples by Tukey's test; PEC, plant extract complex; RF, *Artemisia indica Willd. var. maximowiczii* leaf extract, PTB, *N*-phenacylthiazolium bromide.

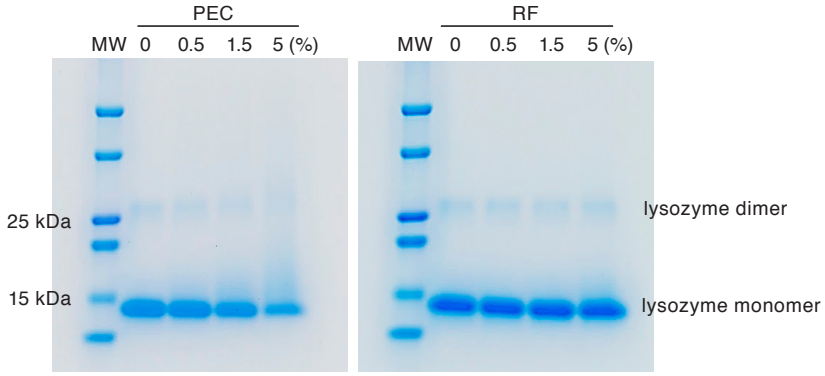

Fig. 4. **CGPC of PEC and RF on lysozyme dimer in the glycated lysozyme.**

Sample and 0.5 mg/mL glycated lysozyme were incubated at 37°C for 16 hours; SDS-PAGE was conducted using 2-40% acrylamide gels. Stained with CBB stain one; CGPC, cleavage of glycated-protein cross-linking; MW, molecular weight markers; BG, butylene glycol; PEC, plant extract complex; RF, *Artemisia indica Willd. var. maximowiczii* leaf extract; 0%, incubation without sample (with 2.5% BG).

Antioxidant activity

The DPPH radical scavenging activity was 13.0-fold higher in PEC (26.0 ± 3.8 mmol-TE/L) than in RF (2.0 ± 0.3 mmol-TE/L, $p < 0.05$, ***Table 4***).

Table 4. Antioxidative activity of PEC and RF.

sample	DPPH radical scavenging activity (mmol-TE/L)	
PEC[1)]	26.0 ± 3.8	*
RF[2)]	2.0 ± 0.3	*

Results are expressed as mean ± standard deviation, n = 3; * $p < 0.05$ by t test; IC_{50}, half maximal inhibitory concentration. 1) plant extract complex, 2) *Artemisia indica Willd. var. maximowiczii* leaf extract. DPPH, 1,1-diphenyl-2-picrylhydrazyl.

Discussion

Inhibitory actions of AGE formation and protein-crosslinking

PEC, RF, and AG were found to inhibit glycation reactions. PEC is a plant extract complex of sea buckthorn fruit, Chinese blackberry tea, tea plants leaves, loquat leaves, and rosemary leaves. The 70% ethanol extract of the leaves and fruits of sea buckthorn has an inhibitory effect on AGE formation in a bovine serum albumin (BSA)-fructose glycation reaction model[38)]. In addition, the fruits of sea buckthorn contain carotenoids, tocopherols, flavonoids, and tannins, which have strong antioxidant effects[39)].

Hot water extract of Chinese blackberry has an inhibitory effect on AGE formation in a collagen-glucose glycation reaction model[40)]. In addition, Chinese blackberry tea contains ellagitannins[41)] and has anti-allergic[42)] and antibacterial[43)] effects. Tea leaves contain catechins[45)]. Catechins have been shown to have antioxidant effects[44)] and inhibitory effects on glycation reactions[40, 46)]. loquat leaves have been shown to inhibit glycation[40)]. Furthermore, corosolic acid contained in loquat leaves has antioxidant[47)] and anti-inflammatory[48)] effects. Rosemary contains rosmarinic acid, which has antioxidant[49)] and anti-inflammatory[50)] effects. Since PEC is a complex, the action of each plant component may act integrally. The taxonomic families of the extract plants that make up the PEC are diverse, with sea buckthorns belonging to the Elaeagnaceae family, Chinese black tea and loquats belonging to the Rosaceae family, tea plants belonging to the Theaceae family, and rosemary belonging to the Lamiaceae family. In plants, the characteristic components of each family are included[51)].

Therefore, PECs may contain multiple phytoconstituents that are not present in a single plant. The glycation reaction system leading to the AGE formation is a complex multi-pathway[17)]. Therefore, multi-component suppression is considered useful for suppressing the formation of AGEs with different pathways[18)]. In the present study, PEC showed stronger inhibition of AGE formation, especially of fluorescent AGEs (***Table 2***), CML, 3DG, GO, and MGO (***Table 3***), in the keratin-glucose glycation model than RF from a single plant.

PEC, RF, and AG all showed inhibitory effects on glycated protein cross-link formation. AGEs include pentosidine, crossline, and glucosepane, which have cross-linking properties. When they accumulate in proteins, they form cross-linked proteins. Some AGEs have fluorescent properties, such as pentosidine[52)], crossline[53)], and pyrropyridine[54)]. The fluorescent and crosslinking properties of AGEs are independent characteristics, with each AGE having both or either. In this study, the inhibitory effect of samples on the formation of dimers and trimers in the lysozyme-glucose glycation reaction model was verified. The inhibitory effect of PEC on glycated protein cross-link formation was stronger than that of RF. However, the IC_{50} value of pentosidine, a type of cross-linking AGEs, was 1.1 times higher with PEC than with RF, which was almost same level. In addition, PEC was 2.8 times stronger than RF in dimer formation inhibition. The difference inhibition effect of dimer formation between PEC and RF suggests that PEC may inhibit the formation of cross-linked AGEs other than pentosidine.

Mugwort, used as a control (RF) for the anti-glycation component, is a plant of the Asteraceae family. An 80% ethanol extract of mugwort has been reported to remove AGEs generated in collagen gel and to improve skin elasticity by application of a formulation containing mugwort extract[25)]. Although the evaluation of the anti-glycation effect in this study was only an *in vitro* study, PEC generally showed stronger effect than RF. The anti-glycation effect of mixed herbal extracts consisting of Dokudami (*Houttuynia cordata*) aerial parts, hawthorn (*Crataegus laevigata*) fruit, Roman chamomile (*Chamaemelum nobile*) flowers and grape (*Vitis vinifera*) leaves was verified both in vitro and in clinical trials[19)]. Ingestion studies of mixed herbal extracts have shown to inhibit the accumulation of AGEs in the blood[20)], improve skin elasticity[21)], inhibit the accumulation of stratum corneum AGEs[22)], and improve dullness[23)]. Mixed herbal extracts are thought to inhibit the formation of a variety of AGEs, with four taxonomically distinct plant species inhibiting multiple AGE formation pathways[19)]. PEC complexes are mixed from the same perspective as mixed herbal extracts and may have similar effects when verified in clinical trials on human subjects.

Cleavage actions against AGE cross-linking and glycated protein cross-linking

PEC and RF showed cleavage effects of AGE cross-linking and glycated protein cross-linking. However, the glycated protein cross-link-cleaving effect of RF showed cleavage rates of less than 20% in the range of 0.5~5% sample concentration, and the cleavage rate was independent of sample concentration. The cleavage activity of AGE cross-linking indicates the cleavage against α-diketone among protein cross-links caused by glycation[10)]. On the other hand, the mechanism of glycated protein cross-link cleavage remains to be elucidated. In addition to α-diketones, glucospan and lysine-dihydropyridinium-lysine have been reported to be involved in the formation of protein cross-links during glycation[10)]. In a study of the AGE cross-link-cleaving and glycated protein cross-link-cleaving effects of 12 substances contained in herbs, no correlation was

observed between the two cleavage effects[33]. The three flavonoids with AGE cross-link-cleaving activity did not show any protein cross-link-cleaving activity[33]. These results indicate that there is no single cross-link structure in the protein molecule cross-linked by glycation. In addition, a single substance involved in cross-link cleavage may not sufficiently cleave the glycated protein cross-links. Based on these results, we speculate that the difference in the action of PEC and RF is due to the action of multiple components in the complex, PEC, other than α-diketones.

The number and intramolecular position of gallate groups attached to flavonoid and tannin molecules are involved in the AGE cross-link cleavage activity of plant components, and the AGE cross-link cleavage activity of substances with trihydroxybenzene structure, the backbone of ellagitannins, is particularly strong[32].

In this study, glycated lysozyme, which was prepared by the lysozyme-glucose glycated reaction model, was used for the glycated protein cross-link cleavage action. The dimer formation rate of the glycated lysozyme was about 12% (results not shown), but the type and number of cross-linked structures were unknown. In order to elucidate the mechanism of the cross-link-cleavage action of glycated proteins, it is necessary to identify the cross-link structures in the glycated proteins and to verify the molecular structures common to substances having the cross-link-cleavage action.

Potential for prevention of skin aging

Proteins in living organisms are constantly exposed to glucose, which exists as a source of cellular energy, and are therefore at risk of glycation[1]. In addition, the AGE formation proceeds due to the progress of glycation reactions and the formation of aldehydes such as 3DG, GO, and MGO associated with the oxidation of glucose[55]. Furthermore, skin tissues are affected by radical formation due to ultraviolet rays in sunlight and oxidation by substances that come in contact with the skin. Oxidation is an accelerator of AGE formation through glycation. Therefore, inhibition of glycation, degradation of AGE cross-links, and suppression of oxidation of skin tissues are important to prevent the accumulation of AGEs.

AGE accumulation in skin tissues has been observed in the dermis[56], epidermis[57,58], and stratum corneum[59]. Glycation of the dermis forms cross-linked structures in collagen and elastin, reducing skin elasticity[58]. Glycation of the stratum corneum disrupts skin texture and accelerates the appearance of aging[60]. The main protein of the stratum corneum is keratin. In this study, PEC was found to inhibit the production of various AGEs on the glycation of keratin and the formation of AGE cross-links. These effects indicate the possibility of suppressing skin aging by applying PEC. In addition, the AGE cross-linking and glycated protein cross-linking cleavage effects of PEC have shown the possibility of being involved in the prevention and improvement of skin tissue elasticity loss. Furthermore, the antioxidant effect inhibits the progression of glycation reactions. These results suggest that the use of skin care formulations containing PEC on skin has potential to act prevention of aging and improvement the functionality of skin tissue.

Research limitations

The plant extracts comprising PEC have already been used as raw materials for cosmetics and are considered to be safe for use in skin care products. On the other hand, the effects of PEC obtained in this study were verified in *in vitro* studies, and the usefulness of PEC for the prevention of aging in skin tissues needs to be verified in clinical studies on humans.

Conclusion

Plant extract complex (PEC) of sea buckthorn fruit, Chinese blackberry tea, tea plant leaves, loquat leaves, and rosemary leaves inhibited the formation of fluorescent AGEs in protein glycation models of HSA, collagen, and keratin. PEC also inhibited the formation of pentosidine, CML, 3DG, GO, and MGO in a keratin-glucose glycation model. Furthermore, PEC inhibited glycated protein cross-linking, cleaved AGE cross-links, and cleaved glycated protein cross-links, suggesting that PEC may be useful in preventing skin aging by suppressing AGE accumulation in skin proteins and preventing protein cross-linking due to glycation.

Declaration of Conflict of Interest

Cocochicosme Co. Ltd. provided research funding support for this study and Cosmo Beauty Co. Ltd. provided samples.

Acknowledgments

Support for the publication of this study was provided by the Isyoku-Dogen Syoyaku Research Foundation.

References

1) Ichihashi M, Yagi M, Nomoto K, et al. Glycation stress and photo-aging in skin. *Anti-Aging Med*. 2011; 8: 23-29.
2) Yagi M, Yonei Y. Glycative stress and anti-aging: 1. What is glycative stress? *Glycative Stress Res*. 2016; 3: 152-155.
3) Yagi M, Yonei Y. Glycative stress and anti-aging: 13. Regulation of glycative stress. 1. Postprandial blood glucose regulation. *Glycative Stress Res*. 2019; 6: 175-180
4) Brownlee M, Vlassara H, Kooney A, et al. Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science*. 1986; 232: 1629-1632.
5) Bolton WK, Cattran DC, Williams ME, et al. Randomized trial of an inhibitor of formation of advanced glycation end products in diabetic nephropathy. *Am J Nephrol*. 2004; 24: 32-40.
6) Hori M, Yagi Y, Nomoto K, et al. Inhibition of advanced glycation end product formation by herbal teas and its relation to anti-skin aging. *Anti-Aging Med*. 2012; 9: 135-148.
7) Ishioka Y, Yagi M, Ogura M, et al. Antiglycation effect of various vegetables: Inhibition of advanced glycation end product formation in glucose and human serum albumin reaction system. *Glycative Stress Res*. 2015; 2: 22-34.
8) Parengkuan L, Yagi M, Matsushima M, et al. Anti-glycation activity of various fruits. *Anti-Aging Med*. 2013; 10: 70-76.
9) Vasan S, Zhang X, Zhang X, et al. An agent cleaving glucose-derived protein crosslinks *in vitro* and *in vivo*. *Nature*. 1996; 382: 275-278.
10) Furber JD. Extracellular glycation crosslinks: prospects for removal. *Rejuvenation Res*. 2006; 9: 274-278.
11) Yagi M, Mitsuhashi R, Watanabe A, et al. Cleaving effect of pomegranate (*Punica granatum*) extract on crosslink derived from advanced glycation endproducts. *Glycative Stress Res*. 2015; 2: 58-66.
12) Takeshita S, Yagi M, Uemura T, et al. Peel extract of water chestnut (*Trapa bispinosa* Roxb.) inhibits glycation, degradesα-dicarbonyl compound, and breaks advanced glycation end product crosslinks. *Glycative Stress Res*. 2015; 2: 72-79.
13) Kawai H, Matsuo N, Yuasa E, et al. Investigation of herbal extracts that have both OPH activity enhancing action and AGE crosslink cleaving activity. *Glycative Stress Res*. 2021; 8: 39-44.
14) Yagi M, Hara H, Mifuru R, et al. Suppression of glycated protein cross-linking formation and cross-linking cleavage reaction of edible purple Chrysanthemum flower extract. *Glycative Stress Res*. 2021; 9: 7-14
15) Takabe W, Mitsuhashi R, Parengkuan L, et al. Cleaving effect of melatonin on crosslinks in advanced glycation end products. *Glycative Stress Res*. 2016; 3: 38-43.
16) Yagi M, Mitsuhashi R, Watanabe A, et al. Cleaving effect of pomegranate (*Punica granatum*) extract on crosslink derived from advanced glycation endproducts. *Glycative Stress Res*. 2015; 2: 58-66.
17) Takeuchi M, Yamagishi S. Involvement of toxic AGEs (TAGE) in the pathogenesis of diabetic vascular complications and Alzheimer's disease. *J Alzheimers Dis*. 2009; 16: 845-858.
18) Yagi M, Yonei Y. Glycative stress and anti-aging: 14. Regulation of Glycative stress. 2. Inhibition of the AGE production and accumulation. *Glycative Stress Res*. 2019; 6: 212-218.
19) Yonei Y, Yagi M, Hibino S, et al. Herbal extracts inhibit Maillard reaction, and reduce chronic diabetic complications risk in streptozotocin-induced diabetic rats. *Anti-Aging Med*. 2008;5: 93-98.
20) Yonei Y, Miyazaki R, Takahashi Y, et al. Anti-Glycation effect of mixed herbal extract in individuals with pre-diabetes mellitus: A double-blind, placebo-controlled, parallel group study. *Anti-Aging Med*. 2010; 7(5): 26-35.
21) Kubo M, Yagi M, Kawai H, et al. Anti-glycation effects of mixed-herb-extracts in diabetes and pre-diabetes. *J Clin Biochem Nutr*. 2008; 43(suppl. 1): 66-69.
22) Yagi M, Shimode A, Hamada U, et al. Evaluation of the anti-glycation effect and the safety of a vinegar beverage containing indigestible dextrin and a mixed herbal extract. A placebo-controlled, double-blind study. *Glycative Stress Res*. 2014; 1: 14-24.
23) Kawai H, Shoshihara M, Kawakami H, et al. Anti-glycation and skin beautification properties from ingestion of mixed herb extract: A placebo-controlled, double-blind, randomized, parallel-group study. *Glycative Stress Res*. 2016; 3: 236-245.
24) Tada A, An evaluation and the material choice of the natural ingredient for anti-glycation cosmetics. *Cosmetic Stage*. 2011; 5: 33-38. (in Japanese)
25) Ohshima H, Tada A, Kanamaru A, et al. Relevance of the directionality of skin elasticity to aging and sagging of the face. *Skin Research and Tech*. 2011; 17: 101-107.
26) Hori M, Yagi M, Nomoto K, et al. Experimental models for advanced glycation end product formation using albumin, collagen, elastin, keratin and proteoglycan. *Anti-Aging Med*. 2012; 9: 125-134.
27) Yagi M, Isshiki K, Takabe W, et al. Measurement of pentosidine in human plasma by the high performance liquid chromatography. *Glycative Stress Res*. 2018; 5: 119-128.
28) Kusunoki H, Miyata S, Ohara T, et al. Relation between serum 3-deoxyglucosone and development of diabetic microangiopathy. *Diabetes Care*. 2003; 26: 1889-1894.
29) Yagi M, Yonei Y. Glycative stress and anti-aging: 2. The Evaluation of glycative stress: Measurement of blood glucose, glycated proteins and intermediates. *Glycative Stress Res*. 2016; 3: 205-209.
30) Yagi M, Yonei Y. Glycative stress and anti-aging. 4: The evaluation of glycative Stress: Evaluation for anti-glycative effect. *Glycative Stress Res*. 2017; 4: 87-92.
31) Vasan S, Zhang X, Zhang X. et al. An agent cleaving glucose-derived protein crosslinks *in vitro* and *in vivo*. 1996; *Nature*. 382: 275-278.
32) Yagi M, Mitsuhashi R, Watanabe A, et al. Cleaving effect of pomegranate (*Punica granatum*) extract on crosslink derived from advanced glycation endproducts. *Glycative Stress Res*. 2015; 2: 58-66.
33) Kim Y, Yokota S, Yagi M, et al. Cleavage of glycated protein cross-linking and cleavage of AGEs cross-linking of substances contained in vegetables and herbs. *Glycative Stress Res*. 2022; 9: 106-111.

34) Perera HKI, Ranasinghe HASK. A simple method to detect plant based inhibitors of glycation induced protein cross-linking. *Asian J Med Sci*. 2015; 6: 28-33.
35) Perera HKI, Handuwalage CS. Analysis of glycation induced protein cross-linking inhibitory effects of some antidiabetic plants and spices. *BMC Complement Altern Med*. 2015; 15, 175.
36) Oki T, Osame M, Masuda M, et al. Simple and rapid spectrophotometric method for selecting purple-fleshed sweet potato cultivars with a high radical-scavenging activity. *Breed Sci*. 2003; 53:101-107.
37) Okuda F, Yagi M, Takabe W, et al. Anti-glycative stress effect of yogurt whey. *Glycative Stress Res*. 2019; 6: 230-240.
38) Lee HHL, Lee CJ, Choi SY, et al. Inhibitory effect of sea buckthorn extracts on advanced glycation endproduct formation. *Food Chemistry*. 2022; 373: 131364.
39) Ciesarováa Z, Murkovic M, Cejpek K, et al. Why is sea buckthorn (*Hippophae rhamnoides* L.) so exceptional? *Food Research Int*. 2020; 133: 109170.
40) Hori M, Yagi M, Nomoto K, et al. Inhibition of advanced glycation end product formation by herbal teas and its relation to anti-akin aging. *Anti-Aging Med*. 2012; 9: 135-148.
41) Tanaka T, Hong ZHANG, Jiang ZZ, Kouno I, et al. Relationship between hydrophobicity and structure of hydrolyzable tannins, and association of tannins with crude drug constituents in aqueous solution. *Chem Pharm Bull*. 1997; 45: 1891-1897.
42) Tanaka, T., Nonaka, G., Nishioka, I., Tannins and related compounds. Part 28. Revision of the structures of sanguiins H-6, H-2, and H-3, and isolation and characterization of sanguiin H-11, a novel tetrameric hydrolysable tannin and seven related tannins from *Sanguisorba officinalis*. *J Chem Research (S)*. 1985, 176-177.
43) Aguilera-Correa JJ, Fernández-López S, Cuñas-Figueroa ID, et al. Sanguiin H-6 fractionated from cloudberry (*Rubus chamaemorus*) seeds can prevent the methicillin-resistant *Staphylococcus aureus* biofilm development during wound infection. *Antibiotics*. 2021; 10: 1481.
44) Rietveld A, Wiseman S. Antioxidant Effects of tea: Evidence from human clinical trials. *J Nutr*. 2003; 133: 3285S-3292S.
45) Tanaka T, Kouno I. Oxidation of tea catechins: Chemical structures and reaction mechanism. *Food Sci Technol Res*. 2003; 9: 128-133.
46) Otake K, Yagi M, Takabe W, et al. Effect of tea (*Camellia sinensis*) and herbs on advanced glycation endproduct formation and the influence of post-fermentation. *Glycative Stress Res*. 2015; 2: 156-162.
47) Li BB, Pang K, Hao L, et al. Corosolic acid improves erectile function in metabolic syndrome rats by reducing reactive oxygen species generation and increasing nitric oxide bioavailability. *Food Sci Technol*. 2022; 42: e108821.
48) Zhao J, Zhou H, An Y, et al. Biological effects of corosolic acid as an anti-inflammatory, anti-metabolic syndrome and anti-neoplasic natural compound. *Oncology Lett*. 2021; 21: 84.
49) Etter SC. *Rosmarinus officinalisas* an antioxidant. *J Herbs Spices Med Plants*. 2005; 11: 121-159.
50) Yua MH, Choia JH, Chae IG, et al. Suppression of LPS-induced inflammatory activities by *Rosmarinus officinalis* L. *Food Chemistry*. 2013; 136: 1047-1054.
51) Robards K, Antolovich M. Analyticalchemistry of fruit bioflavonoids. *Anatyst*. 1997; 122: 11R-34R.
52) Sell DR, Monnier VM. Structure elucidation of a senescence cross-link from human extracellular matrix: Implication of pentoses in the aging process. *J Biol Chem*. 1989; 264: 21597-21602.
53) Obayashi H, Nakano K, Shigeta H, et al. Formation of crossline as a fluorescent advanced glycation end product *in vitro* and *in vivo*. *Biochem Biophys Res Commun*. 1996; 226: 37-41.
54) Hayase F. Recent development of 3-deoxyosone related Maillard reaction products. *Food Science and Technology Res*. 2000; 6: 79-86.
55) Yagi M, Takabe W, Wickramasinghe U, et al. Effect of heat-moisture-treated high-amylose corn starch-containing food on postprandial blood glucose. *Glycative Stress Res*. 2018; 5: 151-162.
56) Mizutari K, Ono T, Ikeda K, et al. Photo-enhanced modification of human skin elastin in actinic elastosis by N(epsilon)-(carboxymethyl)lysine, one of the glycoxidation products of the Maillard reaction. *J Invest Dermatol*. 1997; 108: 797-802.
57) Jeanmaire C, Danoux L, Pauly G, et al. Glycation during human dermal intrinsic and actinic ageing: an *in vivo* and *in vitro* model study. *Br J Dermatol*. 2001; 145: 10-18.
58) Kawabata K, Yoshikawa H, Saruwatari K, et al. The presence of N^{ε}-(carboxymethyl) lysine in the human epidermis. *Biochim Biophys Acta*. 2011; 1814: 1246-1252.
59) Yokota M, Tokudome Y. Permeation of hydrophilic molecules across glycated skin is differentially regulated by the stratum corneum and epidermis-dermis. *Biol Pharm Bull*. 2015; 38: 1383-1388.
60) Gomi T. Evaluation of advanced glycation end products (AGEs) in the stratum corneum and its application. *Bio Industry*. 2011; 28: 20-26. (in Japanese)