Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : September 2, 2017
Accepted : October 26, 2017
Published online : December 31, 2017

Original article

Biochemistry of Kuromoji (*Lindera umbellata*) extract: Anti-oxidative and anti-glycative actions.

Masayuki Yagi [1)], Wakako Takabe [1)], Shigeru Matsumi [2)], Akihiko Shimode [2)], Tetsuya Maruyama [2)], Yoshikazu Yonei [1)]

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

Abstract

***Purpose*:** The purpose of the SIP program in which we participate is to find materials in agricultural products which contain new functions. We furthermore aim to commercialize the products for practical use. This program investigated the biochemical characteristic of kuromoji (*Lindera umbellata*) found through the screening of previous research.

***Method*:** The anti-glycative action, anti-oxidative action and enzyme inhibitory activity of kuromoji extract (test product) were verified. As for anti-glycative activity, the formation inhibition of AGEs (fluorescent AGEs, carboxymethyllysine [CML] and pentosidine) and their intermediates (3-deoxyglucosone [3DG], glyoxal [GO], methylglyoxal [MGO]), the crosslink cleavage activity (α-diketone structure) and activity enhancing action of oxidized protein hydrolase (OPH) at the time when targeted proteins (human serum albumin [HSA], type 1 collagen [Col], elastin [Ela] and glucose [Glu]) were reacted were measured. Anti-oxidative activity was measured by DPPH method and ORAC method. As for enzyme inhibitory activity, the inhibitory activities against α-amylase, α-glucosidase and angiotensin-converting enzyme (ACE) were measured.

***Results*:** The test product showed strong AGE inhibitory activities against not only HSA, but also against Col and Ela, and extensively inhibited the formation of fluorescent AGEs, CML, pentosidine and their intermediates. The crosslink cleavage action and OPH activity enhancing action of the test product were also noted. A strong anti-oxidative activity was shown by DPPH method and ORAC method. The enzyme inhibitory activity showed a strong lipase inhibition, and ACE inhibition and inhibitory activities on α-amylase and α-glucosidase were moderate.

***Conclusion*:** The test product has the actions of AGE formation inhibition, acceleration of AGE decomposition and retardation of digestion, absorption of Glu and fat, and possibly has the function to protect renal function. This test product is to be examined to determine whether it is safe for human use in the future.

***KEY WORDS*:** Kuromoji (*Lindera umbellata*), advanced glycation end products (AGEs), cross-link cleavage, oxidized protein hydrolase (OPH), diabetic nephropathy

Introduction

Since 2014, our laboratory has participated in the program of "Development of Next-Generation Functional Agricultural, Forestry and Fisheries' Products" of "Next Generation Agricultural, Forestry and Fishery Industry Technologies: Creation of Agrinovation," which is the Agriculture, Forestry and Fisheries Department of a national project of "Cross-Ministerial Strategic Innovation Promotion Program (SIP)." The purpose of this program is to find next-generation type new functionalities in the agricultural, forestry and fishery products in Japan, add value to them, and contribute to the activation of agricultural, forestry and fishery industries.

Thus far, our laboratory has searched anti-glycative materials, targeting more than 500 kinds of food materials, by measuring the advanced glycation end products formation inhibitory activities by using *in vitro* glycative reaction models regarding human serum albumin (HSA) as targeted proteins [1-5)]. Furthermore, kuromoji (*Lindera umbellata*) and mugwort (*Artemisia indica* var. *maximowiczii)* were selected as the materials with high characteristic of digestion and absorption and it was verified whether or not they exert their effects on anti-glycative action for diabetic model animals [6)]. Streptozotocin (STZ)-induced diabetic rats were given doses

Contact Address: Professor Yoshikazu Yonei, MD, PhD
Glycative Stress Research Center, Faculty of Life and Medical Sciences, Doshisha University
1-3, Tatara Miyakodani, Kyotanabe, Kyoto, 610-0394 Japan
Phone/Fax: +81-774-65-6394 E-mail: yyonei@mail.doshisha.ac.jp
Co-authors: Yagi M, yagi@mail.doshisha.ac.jp ; Takabe W, wtakabe@mail.doshisha.ac.jp ;
Matsumi S, s-matsumi@yomeishu.co.jp ; Shimode A, a-shimode@yomeishu.co.jp ;
Marutyama T, te-maruyama@yomeishu.co.jp

Glycative Stress Research 2017; 4 (4): 329-340

of kuromoji extract for eight weeks, and their effect on glucolipid metabolic index and the preventive effect on the progression of diabetic nephropathy and cataract were verified. As a result, by dosing kuromoji extract, triglyceride (TG) and free fatty acid (FFA) were improved, inflammatory cytokine in kidney tissue (tumor necrosis factor-α [TNF-α] and interleukin-6 [IL-6]) decreased, kidney function was improved and the preventive effect on progression of cataracts was recognized [6]. For the purpose of social implementation of kuromoji extract, biochemical characteristics such as anti-glycative action, anti-oxidative action and enzyme activity inhibitory action were verified.

Method

Dried and pulverized powder of trunks and branches of kuromoji (*Lindera umbellata*) [6] was used as a test product, which was provided by Yomeishu Seizo Co., Ltd. (Shibuya, Tokyo, Japan).

(1) Test of glycation inhibitory action (human serum albumin reaction system (HSA)

AGEs collectively indicate advanced glycation end products caused by glycation reaction and some AGEs (including pentosidine, crossline and pyrropyridine) exhibit characteristic fluorescence [7]. Glycation inhibitory action was measured by the following procedure: Test product was added to HSA-glucose glycation system and the inhibitory rates of the formation of fluorescent AGEs, 3-deoxyglucosone (3DG), pentosidine and calboxymethyl-lysine (CML) were measured.

As for fluorescent AGEs, as has been previously reported [8], the test samples adjusted to each concentration were added to the solution of *in vitro* glycation 0.1 mol/L NaH_2PO_4-Na_2HPO_4 phosphate buffer (pH 7.4), 8 mg/mL HSA, 0.2 mol/L Glu in order for the concentration of each test material to become 1/10 and were incubated at 60°C for 40 hours. For control purposes, samples in which distilled water was used in place of test product was used. For the measurement of fluorescent AGEs, the fluorescent AGEs formed in the reaction solution after the completion of glycation were measured by a microplate reader (excitation wavelength of 370 nm and fluorescence wavelength of 440 nm).

For the measurement of 3DG, 3DG formed in reaction solution after the completion of glycation was quantified by 2.3-diaminonaphthalen (DAN) pre-labeled reverse phase high performance liquid chromatography (HPLC).

For the measurement of CML, CML formed in the reaction solution was measured by enzyme-linked immunosorbent assay (ELISA) using a measuring kit (CircuLex CML/Nε-(carboxymethyl)lysine, Cylex Incorporated, Ina, Nagano, Japan).

In the case of the measurement of pentosidine by HPLC method, it was measured by reverse-phase HPLC after the reaction solution was hydrolyzed with hydrochloric acid, in reference to the method by Scheijena *et al.* [9].

For the positive control of glycation inhibitory action, aminoguanidine (AG), one of the glycation inhibitors was used.

AGE formation inhibitory rate (%) was calculated from the following equation:

AGE formation inhibitory rate (%) = {1 – (A – B) / (C – D)} × 100

Where A is reaction solution where test material was added at in vitro glycation system, B is that where distilled water was added in place of glucose solution, C is that where only solution without test material was added and incubated, and D is that where distilled water was added in place of glucose as a blank. As anti-AGE activity, IC_{50} (50% inhibition concentration) was calculated.

(2) Test of glycation inhibitory action (Type I collagen [CoI] reaction system)

Glycation inhibitory actions were conducted for 10 days by adding test sample in Gol-Glu glycation system where 1.2 mg/mL of type I collagen derived from cowhide was used in place of HSA, which was used in the HSA reaction system test described above. The formation inhibitory rates of fluorescent AGEs , 3DG and CML by test sample were measured.

(3) Test of glycation inhibitory action (Elastin [Ela])

Glycation inhibitory action was made for 10 days by adding test sample in Ela-Glu glycation system where 6 mg/mL of elastin derived from cowhide was used in place of HSA, which was used in the HSA reaction system test described above.

(4) Test of AGE decomposing action

AGE crosslink cleavage action

N-phenacylthiazolium bromide (PTB) has been reported as a compound that breaks crosslink structure involving AGEs [10]. It is suggested that PTB breaks the C-C bond of α-diketone structure and inhibits the accumulation of AGEs in blood vessels, and as a result, it holds the possibility to contribute to the treatment of diabetic vascular complication. Therefore, attention is paid to the healing approach which may occur for glycative stress. In this test, the AGE crosslink cleavage action was evaluated using a reaction system with model substrate 1-phenyl-1, 2-propanedione (PPD) having α-diketone structure. PTB was used for positive control.

For the measurement of AGE crosslink cleavage action, test material solution or 10 mmol/L PTB, 10 mmol/L PPD and 0.2 mol/L phosphoric acid buffer solution (pH 7.4) were mixed in the proportion of 5 : 1 : 4 and reacted at a 37°C for 8 hours (n = 3). After completion of the reaction, hydrochloric acid was added to the reaction solution and the reaction was ceased. The reaction solution was centrifuged at 20°C for 10 minutes at 3,000 × g, and the volume of benzoic acid in the supernatant was analyzed by reverse phase HPLC. The volume of benzoic acid in the reaction solution was obtained by the amount of benzoic acid in the test sample being deducted, which had been measured separately. As 1 mol of PPD forms 1 mol of benzoic acid, crosslink cleavage rate was calculated by the following equation and the relative value of crosslink cleavage at the time when crosslink cleavage rate of PTB was 100 was regarded as the relative value of cross link cleavage:

Crosslink cleavage rate (%) = {(A – B) / C} × 100

Where A is the amount of benzoic acid in the reaction solution, B is the amount of benzoic acid in test sample and C is the amount of PPD (amount of substrate) provided for reaction.

OPH activity enhancing action

Oxidized protein hydrolase (OPH) is a kind of serine protease releasing N-terminal acylated amino acid of protein which is referred to as acylamino-acid releasing enzyme (AARE) or "acylpeptide hydrolase (APH)" [11]. OPH broadly exists in living tissues such as swine liver, rat brain, human blood and stratum corneum. It is reported that OPH preferentially decomposes oxidized proteins and glycated proteins while at the same time decomposes aged proteins in cooperation with proteasome and decreases amyloid β, one of the causes of Alzheimer's disease [12]. It has also been confirmed that OPH decomposes AGEs. In this measurement, test product solution was added to the reaction system of OPH and N-acetyl-L-alanine p-nitro-anilide (AAPA) which is the reactive substrate of OPH, and the effect of OPH in enzyme reaction was evaluated.

Acylamino-acid releasing enzymes (AARE) and AAPA were used as OPH and reactants for OPH, respectively. OPH was adjusted to 0.01 U/mL, 0.005 U/mL and 0.001 U/mL and used for the measurements. OPH, AAPA and test sample solutions were mixed and put in the wells of a 96-well micro plate and reacted in the incubator set at 37°C for 4 hours, and the light absorption of the reaction solution at 405 nm was measured using a microplate reader. For the enzyme activity, the amount of change of absorbance per hour was obtained. At the same time, the reaction speed at the time when the test sample was not added was obtained as the reference (Ref), and the activity enhancing action at the time when the reaction speed of Ref. was presumed as 100% was calculated by the undermentioned equation. For the control for OPH activity enhancing action, epigallocatechin gallate (EGCg) was used.

OPH activity enhancing action (%) = (OPH reaction speed of test sample/OPH reaction speed of Ref) × 100

(5) Test of anti-oxidative action

Oxidative stress is defined as the difference between the oxidation damaging effects of active oxygen groups formed in vivo and the anti-oxidative potential of anti-oxidative systems in vivo; furthermore, it has involvement in the progress of aging and other various diseases. In this evaluation test, the anti-oxidative actions of the test product were evaluated from various perspectives by the following four methods: the DPPH method using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the ORAC (oxygen radical absorbance capacity) method developed in the US Agriculture Department, the electron spin resonance (ESR) spin trapping method and the anti-oxidative potential (AP) method [13]. For the comparison of anti-oxidative actions, the data were compared with those of vegetables, herbs, tea and healthy tea measured under the same conditions.

The action to eliminate DPPH free radical was measured by the DPPH method. The action to eliminate the free radical formed when 2,2'-azobis (2-amidomp-propane) dehydrochloride was pyrolyzed was measured by the ORAC method. The eliminating activities of superoxide radical, ($O_2 \cdot^-$), hydroxyl radical (HO・) and singlet oxygen ($^1O_2$) were simultaneously measured using ESR apparatus by the ESR spin-trapping method. The above measurements were conducted in Designer Foods Co., Ltd. (Chikusa-ku, Nagoya, Aichi, Japan).

For the measurement of anti-oxidative power by the AP method, when thiocyanic acid compounds and iron (III) ions are mixed, red complexes are formed and the complexes are reduced to iron (II) ions and decolorized by the reduction action of test sample. The changes at this time were measured by the absorbance [13]. Anti-oxidative power is shown as the equivalent amount of ascorbic acid (vitamin C) and the data were compared with those of vegetables, herbs, tea and healthy tea measured under the same conditions. Spotchem i-Pack Oxystress Test (Arkray Co., Nakagyo-ku, Kyoto, Japan) was used for the measurement.

(6) Test of enzyme inhibitory action

Test of amylase inhibitory action

Enzy Chrom α-Amylase Assay Kit (BioAssay System, Hayward, CA, USA) and α-Amylase from Porcine pancreases (Sigma-Aldrich, St. Louis, MO, USA) were used for the discussion on amylase inhibitory action. The enzyme, assay buffer and Glu STD provided as the attachment to the kit were placed in amounts of 10 μL on each well of a microplate. Following this, the working reagents (including test sample solution) corresponding to each test sample in the well were placed in amounts of 40 μL, and the test sample resolutions were stirred and reacted for 15 minutes. After that, 150 μL of a coloring reagent was placed in each well, sample solutions were reacted at room-temperature for 20 minutes and the absorbance at 585 nm was measured. Voglibose of amylase inhibitor was used as positive control of inhibitory action. Amylase inhibitory rate was calculated from the following equation:

Amylase inhibitory rate (%) = (1 – A / B) × 100

Where, A is the absorbance of the test sample and B is that of each solvent (Control: total coloring).

Test of α-glucosidase inhibitory action

Intestinal acetone powder from rats (Sigma-Aldrich) was used for the test of α-glucosidase inhibitory action as inhibitory action QuantiChrom αGlucosidase Assay Kit DAGD-100 (BioAssay System) and α-glucosidase. After the temperature at the measure point of the microplate reader being set at 30°C, 200 μL of a test sample solution was placed on each well and 20 μL of α-glucosidase, assay buffer (pH 7.0), was placed corresponding to each test sample and stirred. Subsequently, the working solution corresponding to the test sample on the well was placed in an amount of 200 μL, and reacted. Absorbance changes at wavelength 405 nm were measured for 30 minutes. For the positive control of inhibitory action, acarbose of α-gulucosidase inhibitors was used. α-Glucosidase was calculated from the following equation:

α-Grucosidase inhibitory rate (%) = (1 – A / B) × 100

Where, A is the absorbance of the test sample and B is that of each solvent (Control: total coloring).

Test of DDP-4 inhibitory action

DPP-4 (dipeptidyl peptidase-4) is a kind of serine peptidase existing on the surface of cells and is an in vivo enzyme which releases X-proline or X-alanine from N-terminal of the peptide chain. DPP-4 highly appears in liver cells, pancreas epithelial cells, intestinal epithelial cells and renal cortex of mammalian tissue. Because DPP-4 plays an important role for glucose homeostasis by the decomposition of incretin such as GLP-1, it is paid attention to as the new drug discovery target of type 2 diabetes. In this method, DPP-4 activity was measured by the measurement of fluorescence intensity emitted when fluorescently labeled DPP-4 substrate is decomposed. For the positive control,

P32/98-competing drug, which is a DPP-4 inhibitor, was used. SensoLyte Rh110 DPPIV Assay Kit (AnaSpec, Fremont, CA, USA) was used for the measurement.

In accordance with the procedures described for the kit, test samples of 10 μL and enzyme diluents of 40 μL were put in the wells of microplates, and incretin 50 μL of a substrate solution incubated at 37°C was added to each well. In turn, the fluorescence intensities at an excitation wavelength of 492 nm and at fluorescence wavelength of 533 nm were measured at 37°C for 60 minutes using a microplate reader. DPP-4 inhibitory rate was calculated from the following equation:

DPP inhibitory rate (%) = (1 – A / B) × 100

Where, A is the absorbance of the test sample and B is that of each solvent (Control: total coloring).

Test of angiotensin converting enzyme (ACE) inhibitory action

Angiotensin-converting enzyme (ACE) is an enzyme that transforms angiotensin I to angiotensin II at renin-angiotensin systems. In this measurement, ACE inhibitory activity was measured from the amount of 3-hydroxybutyric acid (3HB) that was released from 3-hydroxybutyryl-Gly-Gly-Gly (3HB-GGG) by ACE. For positive control, captopril, an angiotensin-converting enzyme inhibitor, was used. ACE-Kit WST (Dojin Chemical, Kamimashiki-gun, Kumamoto, Japan) was used for measurement.

In accordance with the procedures described for this measuring kit, 20 μL of test sample, 20 μL of substrate buffer and 20 μL of enzyme working solution were placed in each well of microplates and were incubated at 37°C for 60 minutes. After that, 200 μL of an indicator working solution was placed in each well and the solutions were incubated at room temperature for 10 minutes. Following this, the absorbance at 450 nm were measured using a microplate reader. ACE inhibitory rate was calculated from the following equation:

Angiotensin-converting enzyme inhibitory rate (%) = (1 – A / B) × 100

Where, A is the absorbance of the test sample and B is that of each solvent (Control: total coloring).

Test for lipase inhibitory action

Lipase is a group of enzymes which breaks ester bond constituting lipids and is involved in the decomposition of lipids. In this testing method, the activity of lipase was measured by the measurement of fluorescence intensity of 4-methylumbelliferone formed through the reaction of oleic acid ester of 4-methylumbelliferon and lipase. For positive control, minocycline was used, which is one of the tetracycline anti-biotic agents having an anti-lipase action. For substrate solution, 50 μL of 0.1 mmol/L 4-methylumbeliferyl olate, 25 μL of test sample and 25 μL of lipase solution of 1.25 mg/mL were mixed and incubated at 30°C for 15 minutes. After that, 100 μL of citrate buffer solution of 0.1 mol/L (pH 4.2) was added to stop enzyme reaction, 100 μL of the reaction solutions was placed in each well of the 96-well black microplate and their fluorescence intensities at an excitation wavelength of 360 nm and fluorescence wavelength of 460 nm were measured using a microplate reader. Lipase inhibitory rate was calculated from the following equation:

Lipase inhibitory rate (%) = (1 – A / B) × 100

Where, A is the absorbance of the test sample and B is that of each solvent (Control: total coloring).

Result

Glycation action: AGE and intermediate formation inhibition

Table 1 shows the results of the comparison of anti-glycative activity of test product (kuromoji extract) with the positive control of AG.

The test product (1 mg/mL) inhibited the formation of fluorescent AGEs, 3DG, GO and CML in HSA-Glu reaction system more than 80%. It inhibited the formation of fluorescent AGEs, 3DG, GO, CML and pentosidine in Col-Glu reaction system more than 80%. It also inhibited the formation of 3DG, GO, MGO and CML in Ela-Glu reaction system more than 80%.

Positive control AG (1 mg/mL) inhibited the formation of fluorescent AGEs, 3DG and GO in HSA-Glu reaction system more than 80%. It inhibited the formation of GO and CML in Col-Glu reaction system more than 80%. It inhibited the formation of MGO only in Ela-Glu reaction system.

In HSA-Glu reaction system, fluorescent AGE and 3DG formation inhibitory actions of the test product were equivalent to those of AG (IC_{50} ratio: fluorescent AGEs 0.9, 3DG 1.5), and CML formation inhibitory action of the test product (IC_{50} ratio: 4.1) was stronger than that of AG (***Fig. 1***).

In Gol-Glu reaction system, fluorescent AGE, 3DG and CML formation inhibitory actions of test product were stronger than those of AG (IC_{50} ratio: fluorescent AGEs 13.4, 3DG 9.5 and CML 4.6, ***Fig. 2***).

In the Ela-Glu reaction system, the fluorescent AGE formation inhibitory action of the test product was equivalent to that of AG IC_{50} ratio: 2.3), and 3DG and CML formation inhibitory actions of the test product were stronger than those of AG (IC_{50} ratio: 3DG 15.7 and CML 46.9, ***Fig. 3***).

Anti-glycative action: AGE decomposition promotion

As for crosslink cleavage action of the test product, an equivalent of 1 mg/dL in kuromoji extract is approximately 30 percent of the positive control PTB (relative value 34) and was approximately identical to PTB (relative value 97) at high dosage (5 mg/dL) (***Table 2***).

OPH activity enhancing action of the test product was recognized to have 146.3 ± 1.1% when presumed as 100% when the test product was not added in high dosage (5 mg/dL) (***Table 3***).

Anti-oxidative action

Anti-oxidative action of the test product was examined using the DPPH method and ORAC method at high dosages of kuromoji extract (10 mg/dL), and the relative values of superoxide scavenging activity and singlet oxygen scavenging activity were 72.4-164.9, which were similar to those of green perilla (*Perilla frutescens*) when assumed as 100 (***Table 4***). The relative values of hydroxyl radical scavenging activity and vitamin C content were low when green perilla was assumed as 100 (relative value: hydroxyradical scavenging activity 14 and vitamin C content 14.1). By evaluation via the AP method, the result of the kuromoji extract high-dose (10 mg/dL) (vitamin equivalent: 5217 ± 226 mg/L) was similar to yuzu citrus (***Table 5***).

Table 1. Inhibitory effect of Kuromoji extract on AGE and intermediate formation.

Index for anti-glycative actions	Target protein	Test product		Positive control		IC_{50} ratio (Positivecontrol / Test product)
		Kuromoji extract		Aminoguanidine		
		% Inhibition (1 mg/mL) (%)	IC_{50} (mg/mL)	% Inhibition (1 mg/mL) (%)	IC_{50} (mg/mL)	
Fluorescent AGEs	HSA	80.4	0.127	98.4	0.111	0.9
	Col	92.5	0.029	69.0	0.393	13.4
	Ela	66.2	0.286	64.5	0.659	2.3
3DG	HSA	81.9	0.177	93.8	0.257	1.5
	Col	81.6	0.223	36.9	2.116	9.5
	Ela	80.8	0.128	42.6	1.999	15.7
GO	HSA	83.8	0.145	92.2	0.108	0.7
	Col	88.5	0.283	88.0	0.145	0.5
	Ela	120.0	0.152	17.5	> 1.0	NC
MGO	HSA	76.0	0.262	77.8	0.409	1.6
	Col	65.6	0.380	71.9	0.314	0.8
	Ela	95.9	< 0.0001	92.7	< 0.001	NC
CML	HSA	98.4	0.064	76.3	0.261	4.1
	Col	98.4	0.027	87.8	0.124	4.6
	Ela	104.9	0.031	47.6	1.439	46.9
Pentosidine	HSA	51.1	0.553	–	–	–
	Col	93.2	ND	–	–	–

AGEs, advanced glycation end products; IC_{50}, 50% inhibitory concentration; 3DG, 3-deoxyglucosone; GO, glyoxal; MGO, methylglyoxal; CML, carboxymethyl-lysin; HSA, human serum albumin; Col, collagen type I; Ela, elastin; NC, not caliculated.

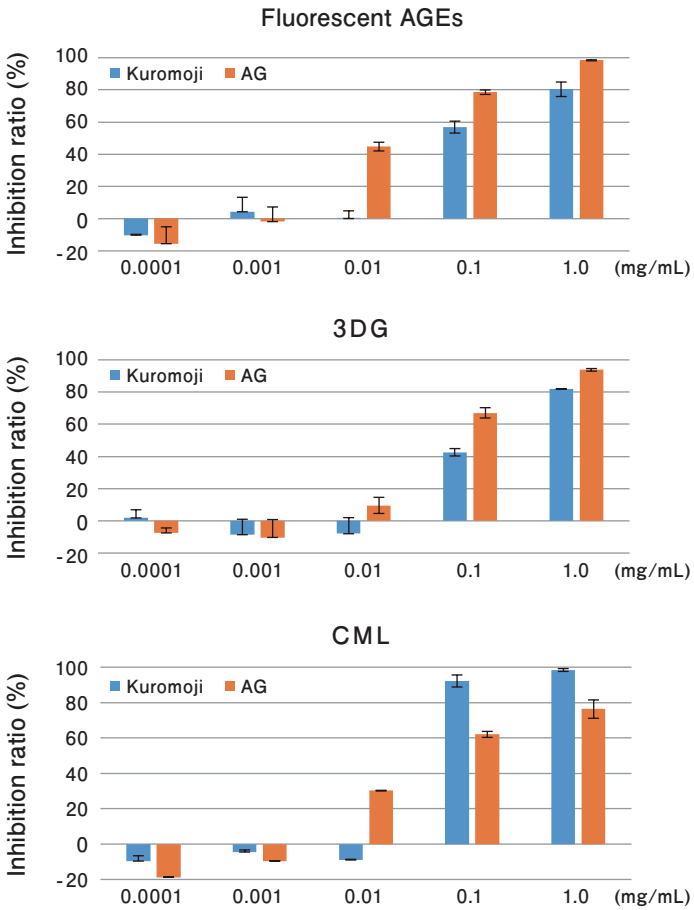

Fig. 1.

Inhibitory effect of Kuromoji extract on AGE and intermediate formation in the HSA-Glu reaction model.

a) Fluorescent AGEs, **b)** 3DG, **c)** CML. Results are expressed as mean ± SD (n = 3). AGEs, advanced glycation end products; HSA, human serum albumin; Glu, glucose; AG, aminoguanidine; 3DG, 3-deoxyglucosone; CML, carboxymethyl-lysin; SD, standard deviation.

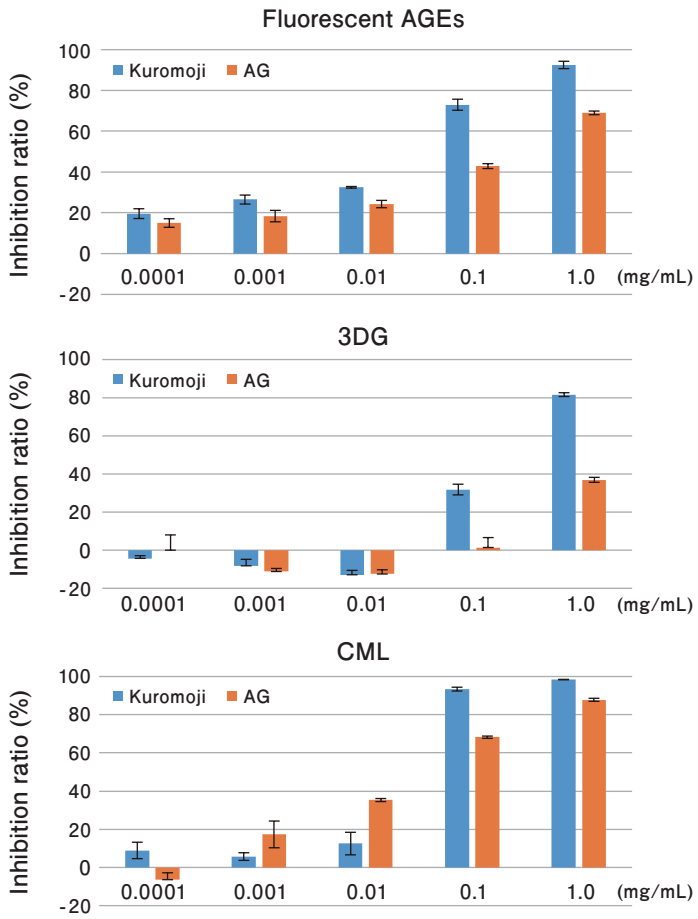

Fig. 2.

Inhibitory effect of Kuromoji extract on AGE and intermediate formation in the Col-Glu reaction model.

a) Fluorescent AGEs, **b)** 3DG, **c)** CML. Results are expressed as mean ± SD (n = 3). AGEs, advanced glycation end products; Col, collagen type I; Glu, glucose; AG, aminoguanidine; 3DG, 3-deoxyglucosone; CML, carboxymethyl-lysin; SD, standard deviation.

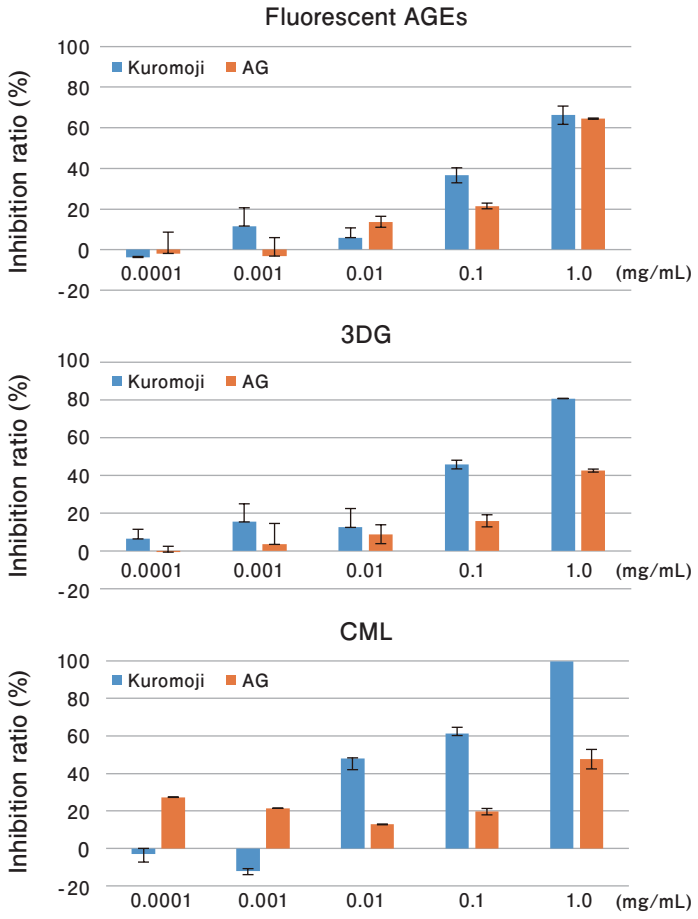

Fig. 3.

Inhibitory effect of Kuromoji extract on AGE and intermediate formation in the Ela-Glu reaction model.

a) Fluorescent AGEs, **b)** 3DG, **c)** CML. Results are expressed as mean ± SD (n = 3). AGEs, advanced glycation end products; Ela, elastin; Glu, glucose; AG, aminoguanidine; 3DG, 3-deoxyglucosone; CML, carboxymethyl-lysin; SD, standard deviation.

Table 2. AGE cross-link breaking activity

Sample	Concentration (mg/mL)	Perecent breakage (%)	Relative value
Kuromoji extract (Test product)	0.008	2.7 ± 0.03	11
	0.04	2.9 ± 0.02	12
	0.2	3.7 ± 0.01	16
	1.0	8.1 ± 0.04	34
	5.0	23.1 ± 0.17	97
PTB (Positive control)	5 mmol/L	23.9 ± 0.1	100

Results are expressed as mean ± SD (n = 3). AGE, advanced glycation end product; PTB, N-phenacylthiazolium bromide; SD, standard deviation.

Table 3. OPH enhance activity.

Sample	Concentration (mg/mL)	Percent enhancement (%)
Kuromoji extract (Test product)	5.0	146.3 ± 1.1
Control (no reagent added)	–	100.0
EGCg (Positive control)	1.0	15.5 ± 3.2

Results are expressed as mean ± SD (n = 3). OPH, oxidized protein hydrolase; EGCg, epigallocatechin gallate; SD, standard deviation.

Table 4. Anti-oxidative profile of kuromoji extract.

Method	Value	Unit	Relative value [1)]
DPPH	12,900	mg TE/100g	72.4
ORAC	376,000	mg TE/100g	131.1
ESR (Superoxide scavenging activity)	37,800	unit SOD/g	115.9
ESR (Hydroxyl radical scavenging activity)	53,400	μmol DMSO/g	14
ESR (Singlet oxygen scavenging activity)	218,000	μmol Histidine/g	164.9
Sugar content	1.9	%	0.2
Vitamine C content	888	mg/100g	14.1
Nitrate ion content	2,610	mg/kg	5.8

1) The relative value when assuming the megaphylly (Jp; ouba, En; green perilla, Sc; *Perilla frutescens*) as 100. DPPH, 1,1-diphenyl-2-picrylhydrazyl; ORAC, oxygen radical absorbance capacity; TE, trolox equivalent; ESR, electron spin resonance; SOD, super oxide dismutase; DMSO, dimethyl sulfoxide; Jp, Japanese name; En, English name; Sc, scientific name.

Table 5. AP method.

Extract sample	Anti-oxidant potential (mg VC Eq/L)
Kuromoji [En, JP], Lindera [En], , *Lindera umbellata* [Sc] (10 mg/mL)	5,217 ± 226
Green tea [En], Ryoku-cha [Jp], *Camelia sinensis* [Sc] (products of Kagoshima)	3,151
Black tea (leaf) [En], Kou-cha (ha) [Jp], *Camelia sinensis* [Sc]	1,929
Coarse tea [En], Ban-cha [Jp], *Camelia sinensis* [Sc]	1,592
Pu-erh tea [En], Puaru tea [Jp], *Camelia sinensis* [Sc]	1,590
Yuzu [En, Jp], *Citrus junos* [Sc]	5,749
Sudachi [En, Jp], *Citrus sudachi* [Sc]	3,618
Lemon [En, Jp], *Citrus limon* [Sc]	3,614
Fukinoto [En, Jp], *Petasites japonicas* [Sc]	3,615
Edible chrysanthemum [En], Shokuyo-kiku [Jp], *Chrysanthemum* × *morifolium f. esculentum* [Sc]	2,100
Chestnut (astringent skin) [En], Kuri (shibu kawa) [Jp], *Castanea crenata* [Sc]	2,071

Results are expressed a single-measued value (n = 1) except for kuromoji in which the value shows mean ± SD (n = 4). Anti-oxidant potential is expressed as VC equivalent (Eq). Hot water extracts were prepared from each dried sample of vegetable, fruits, tea and herbs (4 g in 40 mL hot water) at 80 ° C for 1 hour. AP, anti-oxidant potential; VC, vitamin C; SD, standard deviation; Jp, Japanese name; En, English name; Sc, scientific name.

Enzyme inhibitory action

Although α-amylase inhibitory action (IC_{50}: 2.04 mg/mL) and α-glucosidase inhibitory action (IC_{50}: 1.06 mg/mL) were recognized in kuromoji extract, no DPP-4 inhibitory actions were observed (***Table 6***). ACE inhibitory action was strong (IC_{50}: 0.29 mg/mL). Regarding protein and fat decomposing enzyme, lipase inhibitory action was strong (IC_{50}: 0.022 mg/mL).

Discussion

Modern society has reached an era of over-eating and lack of exercise. Glucose absorbed into the body is not used in skeletal muscles enough yet, instead, is stored in fat tissue and the liver. Moreover, the terminal aldehyde group of glucose reacts with structural proteins and functional proteins and causes various results (glycation). Furthermore, glyceraldehyde derived from the reaction of glycolysis, acetaldehyde caused by drinking, aldehydes derived from lipids including TG and low-density lipoprotein cholesterol (LDL-C) and aromatic aldehyde derived from fragrances are responsible for glycation. Glycative stress is the condition where aldehydes are easily generated in the body, which are responsible for diabetes, dyslipidemia, metabolic syndrome, and even chronic kidney disease (CKD). Aldehydes react with proteins in the body, promote the formation and accumulation of AGEs, generate inflammatory cytokines through RAGE (receptor for AGEs) signal activity, and as a result, easily lead to problems such as tissue damage.

Glycative stress is divided into the stages of (1) prevention of postprandial hyperglycemia, (2) prevention of the formation of AGEs, (3) promotion of decomposition and evacuation of AGEs, and (4) prevention of AGEs and RAGE signal activities [14]. As oxidative stress accelerates the formation reaction of AGEs; therefore, it is important to pay attention to oxidative stress. Glucose is often given more attention than glycative stress; however, attention must be paid to excess lipids as well in the case of diabetes.

AGE and intermediate formation inhibition

The test product (kuromoji [*Lindera umbellata*] extract) showed a strong ability for "(2) prevention of the formation AGEs." Its anti-glycative activity against the target protein Col (AGE and intermediate formation inhibition) was strong and strongly inhibited the formation of fluorescent AGEs, 3DG, GO, MGO, CML and pentosidine. Similarly, kuromoji extract strongly inhibited the formation of 3DG, GO, MGO and CML of the target protein Ela. Its effect on HSA was also observed to be strong as AG of the positive control or more in inhibiting the formation of AGEs, 3DG, GO, MGO and CML. As the characteristics of the test product, it exerts its preventive action against the glycation of extensive target proteins, and in particular, it has the ability to prevent the glycation of proteins involved in the structure and function of skin and joints such as Col and Ela. It is a material suitable to the SIP program which is developing new functional food relating to locomotive syndrome.

Table 6. Enzyme inhibition assay.

a) α-Amylase

Sample	Concentration (mg/mL)	Percent inhibition (%)			IC_{50} (mg/mL)
		# 1	# 2	Average	
Kuromoji extract (Test product)	0.016	−45.6	−50.5	−48.0	2.04
	0.08	−89.7	−92.7	−91.2	
	0.8	14.2	21.4	17.8	
	1.6	41.7	40.3	41.0	
	4.0	70.1	76.8	73.5	
Voglibose (Positive control)	0.008	46.7	45.3	46.0	—

b) α-Glucosidase

Sample	Concentration (mg/mL)	Percent inhibition (%)			IC_{50} (mg/mL)
		# 1	# 2	Average	
Kuromoji extract (Test product)	0.018	−3.6	7.0	1.7	1.06
	0.09	13.9	13.1	13.5	
	0.9	43.6	35.8	39.7	
	1.8	60.3	61.2	60.7	
	4.4	70.4	73.3	71.9	
Acarbose (Positive control)	0.01	47.9	45.0	46.5	—

c) DPP-4

Sample	Concentration (mg/mL)	Percent inhibition (%)			IC_{50} (mg/mL)
		# 1	# 2	Average	
Kuromoji extract (Test product)	0.01	17.8	—	18.0	ND
	0.1	−3.0	1.4	−0.8	
	0.2	4.3	0.6	2.5	
	0.5	23.6	−4.1	9.8	
	1.0	11.6	3.5	7.5	
P32/98 (Positive control)	0.1μmol/L	52.1	—	52.1	—

d) ACE

Sample	Concentration (mg/mL)	Percent inhibition (%)			IC_{50} (mg/mL)
		# 1	# 2	Average	
Kuromoji extract (Test product)	0.033	10.7	6.9	8.8	0.290
	0.07	14.9	15.8	15.3	
	0.3	53.1	48.1	50.6	
	0.7	67.9	70.6	69.3	
	1.7	87.2	86.6	86.9	
Captopril (Positive control)	0.0006	44.5	57.5	51.0	—

e) Lipase

Sample	Concentration (mg/mL)	Percent inhibition (%)			IC_{50} (mg/mL)
		# 1	# 2	Average	
Kuromoji extract (Test product)	0.00025	– 8.3	±	18.9	0.022
	0.0025	15.2	±	9.0	
	0.025	61.5	±	0.7	
	0.25	77.7	±	4.4	
Minocyclin (Positive control)	0.5 mmol/L	77.2	±	2.4	—

Results are expressed as mean ± SD, n = 2 or 3. IC_{50}, 50% inhibitory concentration; DPP-4, dipeptidyl peptidase-4; ACE, angiotensin-converting enzyme; SD, standard deviation; ND, not determined.

AGE decomposition promotion

AGE crosslink cleavage action and OPH activity enhancing action are related to "(3) promotion of decomposition and evacuation of AGEs." The materials known to have AGE crosslink cleavage action are gallic acid contained in pomegranate (*Punica granatum*), hydroxyquinol and pyrogallol of hydroxyl benzene compounds, hydroquinone and pyrocatechol of dihydroxybenzene compounds [15], ellagitannin included in water chestnut (*Trapa natans*) [16], iridoid compounds included in noni (*Morinda citrifolia*), and Japanese cornel (*Cornus officinalis*) and olive (*Olea europaea*) [17]. Melatonin, a hormone secreted during sleep, which also exists in vegetables, strengthens the AGE crosslink cleavage action [18]. AGE decomposition promotion action that kuromoji has is moderate, although slightly weaker than that which *Punica grantaum* and *Trapa natans* have; it is presumed to synergistically act on the reduction of glycative stress.

It has previously been confirmed that OPH has an AGE decomposition action [11]. A substance that strengthens OPH activity is assumed to exist. In this test, it was shown that kuromoji extract (5 mg/nL) strengthens OPH activity by 46%. Theoretically, this action works in sync with the alleviation of glycative stress.

Anti-oxidative action

Generally speaking, oxidative stress accelerates the complicated responses concerning glycation though various routes. Because GO and MGO of intermediates are formed by automatic oxidation, the anti-oxidative substances act on these routes in a suppressive way. GO, which reacts with lysine, is involved in the formation of CML [7], and because the oxidation reaction is involved in this process, when oxidative stress is strengthened, the formation of CML increases. CML is one of non-fluorescent AGEs and is formed by oxidative stress and increasing oxidative stress [19]. A strong anti-oxidative action was recognized in kuromoji extract by the evaluation of anti-oxidative actions in this study. This action is expected to act on the formation of AGEs in an inhibitory way.

Enzyme inhibitory activity

Carbohydrate substances contained in food are decomposed into Glu, which is a single sugar, and others by digestive enzymes, which are absorbed from the intestine. Amylase breaks down carbohydrate substances into double sugar in digestive intestine. Similarly, α-glucosidase breaks

down double sugar into a single sugar. The materials having amylase inhibitory action are taken together with food so that they may inhibit the breakdown of carbohydrates in food and slow down postprandial hyperglycemia. In this test, α-amylase inhibitory action was confirmed using amylase derived from porcine pancreases.

Drastic elevation of glucose in blood encourages postprandial hyperglycemia, and during that time, eases the progression of glycation reaction. Due to the inhibition of enzyme action, it can make the absorption of Glu to blood vessels through the small intestine moderate, so that it is positioned in the measures against glycative stress as "(1) prevention of postprandial hyperglycemia." In this test, although their effects were not as strong as *Geranium dielsianum* extract (IC_{50}: 0.028 mg/dL) [20], α-amylase inhibitory action (IC_{50}: 2.04 mg/dL) and α-glucosidase inhibitory action (IC_{50}: 1.06 mg/dL) were both found in moderate levels. Extract of kuromoji has the possibility to moderate postprandial hyperglycemia.

The aldehydes responsible for glycation are not only derived from reducing sugars such as Glu and fructose, but also lipids such as TG and LDL-C. Lipase is an enzyme that breaks down fat and assists in its digestion and absorption. The substances having lipase inhibitory actions inhibit the breakdown of fat as well as the absorption of fat. In this research, a strong lipase inhibitory action was recognized in the test product (IC_{50}: 0022 mg/dL). In our precedent research, kuromoji extract was given to STZ-induced rats for eight weeks [6]. As a result, significant improvements were observed in TG and FFA [6]. As its mechanism, lipase inhibitory action has the possibility for high involvement.

ACE is an enzyme which forms angiotensin II having hypertensive action from angiotensin I in rennin-angiotensin system, which is one of the human blood pressure adjustment mechanisms. At the same time, it has the action to breakdown bradykinin, which is an anti-hypertensive peptide, and is greatly involved in blood pressure elevation in this manner. Angiotensin II causes renal artery constriction and brings about the action to lower renal blood flow and renal filtration volume (lowering of creatinine clearance [CCr]). This research showed that the test product had ACE inhibitory action (IC_{50}: 0.29 mg/mL). In our precedent research, as the result that kuromoji extract was dosed to STZ-induced rats for eight weeks, the lowering of CCr in association with diabetic nephropathy was significantly improved [6]. It is presumed that kuromoji not only exerted its anti-glycative action, but also maintained renal blood flow through inhibiting the rising of angiotensin II by ACE inhibitory action. As a result, it led to renal protection.

Conclusion

Through examining the anti-glycative action and anti-oxidative action of the test product (kuromoji [*Lindera umbellata*] extract) selected through screening of food materials, the test product showed strong AGE formation inhibitory activity not only in HSA but also in Col and Ela, and it showed moderate AGE decomposition promotion activity and strong anti-oxidative activity. As for the enzyme inhibitory activity, α-amylase inhibitory action, α-glucosidase inhibitory action and lipase inhibitory action were shown and may possibly alleviate postprandial high blood glucose and postprandial hypertriglyceridemia, In addition, ACE inhibitory action may assist in protecting renal functions. This test product is to be examined for safety and efficacy evaluation for human use in the future.

Acknowledgement

This work was supported by the Japanese Council for Science, Technology and Innovation, SIP (Project ID 14533567), "Technologies for creating next-generation agriculture, forestry and fisheries" (funding agency: Bio-oriented Technology Research Advancement Institution, NARO).

Conflict of Interest Statement

The present study was supported by a SIP cooperative company, Yomeishu Seizo Co. Ltd.

Reference

1) Hori M, Yagi M, 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.
2) Parengkuan L, Yagi M, Matsushima M, et al. Anti-glycation activity of various fruits. Anti-Aging Med. 2013; 10: 70-76.
3) 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.
4) Tanaka Y, Yagi M, Takabe W, et al. Anti-glycative effect of yogurt: Prevention of advanced glycation end product formation. Glycative Stress Res. 2017; 4: 25-31.
5) Takabe W, Kitagawa K, Yamada K, et al. Anti-glycative effect of vegetables and fruit extract on multiple glycation models. Glycative Stress Res. 2017; 4: 71-79.
6) Yagi M, Takabe W, Matsumi S, et al. Screening and selection of anti-glycative materials: Kuromoji (*Lindera umbellata)*. Glycative Stress Res. 2017; 4: 317-328
7) Nagai R, Mori T, Yamamoto Y, et al. Significance of advanced glycation end products in aging-related disease. Anti-Aging Med. 2010; 7: 112-119.
8) Hori M, Yagi M, 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.

9) Scheijen JL, van de Waarenburg MP, Stehouwer CD, et al. Measurement of pentosidine in human plasma protein by a single-column high-performance liquid chromatography method with fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2009; 877: 610-614.
10) 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.
11) Yagi M, Ishigami M, Mori R, et al. Eliminating effect of oxidized protein hydrolase (OPH) on advanced glycation end products and OPH-like activity in human stratum corneum. Glycative Stress Res. 2017; 4: 184-191.
12) Yagi M, Yonei Y. Glycative stress and anti-aging: 1. What is glycative stress? Glycative Stress Res. 2016; 3: 152-155.
13) Sato K, Yagi M, Yonei Y. A new method for measuring oxidative stress using blood samples. Glycative Stress Res. 2015; 2: 15-21.
14) Ichihashi M, Yagi M, Nomoto K, et al. Glycation stress and photo-aging in skin. Anti-Aging Med. 2011; 8: 23-29.
15) 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.
16) 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.
17) Abe Y, Yagi M, Uwaya A, et al. Effect of iridoid (containing plants) on AGE formation and degradation. Glycative Stress Res. 2016; 3: 56-64.
18) 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.
19) Nagai R, Unno Y, Hayashi MC, et al. Peroxynitrite induces formation of N^{ε}-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: Novel pathways for protein modification by peroxynitrite. Diabetes. 2002; 51: 2833-2839.
20) Takahashi K, Nomoto K, Ito M, et al. In vitro effects of *Geranium dielsianum* extract on glycative stress. Glycative Stress Res. 2015; 2: 208-216.