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# Original article Inhibitory effect of natural products on the formation of advance glycation end products.

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## Abstract

Advanced glycation end products (AGEs) is a generic name for compound produced by a non-enzymatic reaction of the Maillard reaction between proteins and reducing sugars. In the reaction, AGEs generate in a variety of proteins *in vivo* such as hemoglobin, albumin and collagens. Moreover, as we grow older, AGEs are accumulated especially in long-life proteins, which is comparatively slow to be metabolized, and it is reported that life-style related diseases based on dietary habits or lack of exercises accelerate AGE accumulation. The aim of this study is to prevent life-style related diseases by daily intake of AGE inhibitors in foods. Natural materials were extracted by several organic solvents, such as ethanol and acetone. The extracts were filtrated and dried in vacuo, and incubated them for a week at a temperature of 37°C by adding them into a ribose/gelatin solution. After that, inhibitory effect of AGE formation was measured based on ELISA, using monoclonal antibodies against  $N^{\varepsilon}$ -(carboxymethyl)lysine (CML), which is one of oxidation-dependent AGEs, or  $N^{\omega}$ -(carboxymethyl) arginine (CMA), which is one of collagen specific AGEs. As results, three kinds of lamiaceae plants inhibited the formation of both CMA and CML suggesting that lamiaceae specific compounds including rosmarinic acid may play a role in inhibiting AGE formation. Taken together, these results suggest that a daily intake of these natural materials may prevent AGE formation and is expected to show an anti-aging effect, as well as effects to prevent various diseases including arteriosclerosis and cataracts.

**KEY WORDS:** advanced glycation end products (AGEs), glycation, natural products, lifestyle-related diseases, diabetes mellitus

# Introduction

The number of diabetic patients in the world is continuously increasing. In 2014, it reached about 422 million, which is reported to be approximately four times the figure in 1980<sup>1</sup>). Currently, a measurement of Hemoglobin A1c (HbA1c), which is formed by the reaction between hemoglobin and blood glucose, is generally adopted as a clinical marker of blood glucose control in patients with diabetes. The nonenzymatic reaction of protein and blood reducing sugar is called Maillard reaction or glycation, and is seen not only with hemoglobin but also other various proteins such as albumin or collagen. Eventually, advanced glycation end products (AGEs) are formed through oxidation, dehydration and condensation. It is reported that AGEs accumulate in our body over time, and the accumulation is progressed due to the diseases caused by lifestyle including excessive calorie intake and insufficient exercise. In order to reduce the physical influence of AGEs, there are three methods: 1)

Address for correspondence: Dr. Ryoji Nagai Laboratory of Food and Regulation Biology, Graduate School of Agriculture,Tokai University, Toroku,Higashiku 9-1-1, Kumamoto, Kumamoto 862-8652, Japan TEL&FAX: +81-096-386-2692 Email: nagai-883@umin.ac.jp Co-authors: Kinoshita S, 6bnfm003@mail.u-tokai.ac.jp; Furusawa C, indiog\_light@yahoo.co.jp; Sugawa H, rice\_skydog@yahoo.co.jp; Shirakawa J, jshirakawa33920@gmail.com; Ohno R, reiichi296@gmail.com; Ichimaru K, 6bnfm002@mail.u-tokai.ac.jp; Nagai M, komime@gmail.com; AGE decomposition by AGE-breaker <sup>2)</sup>, 2) an antagonistic inhibition of AGE receptors <sup>3)</sup>, and 3) inhibition of AGE formation <sup>4)</sup>. However, in fact it is difficult to decompose the accumulated AGEs by using medicines <sup>5)</sup>. In addition, regarding the development of AGE receptor competitive inhibitors, *in vitro* evaluation of the AGE receptor function has not yet been verified. Therefore, an experiment using an animal is indispensable in the early stages.

For this reason, various research has been conducted on the prevention of AGE accumulation *in vivo* by inhibiting AGE formation, as seen in 3). Currently, aminoguanidine (AG) is the prototype and well-known inhibitor for AGE formation. It is reported that AG inhibits the formation of 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), and prevent crosslink in collagen due to glycation<sup>5</sup>). However, due to the side effects such as anemia and nephritis confirmed after the clinical trial, AG is not applied for practical use<sup>6</sup>).

Based on this circumstance, AGE inhibitors derived from

natural products have been studied worldwide. Although many studies reported that natural products inhibit AGE formation, those detected AGE structures have not identified yet. Furthermore, it is well known that the structures of accumulated AGEs vary depending on each disease, as AGEs are generated in several pathways with different kinds of carbohydrates and amino acids. Based on this, it is important to measure AGEs for each structure so that their features or relationships with diseases can be clarified.

So far, our laboratory reported that the level of pentosidine in the human serum is inhibited by oral administration of mangosteen pericarp extracts, resulting in an improvement of the elasticity and moisture content of the skin<sup>7</sup>. Moreover, our previous study also demonstrated that  $N^{\varepsilon}$ -(carboxyethyl) lysine (CEL) is generated from ketone body, and supplementation of citric acids to type l diabetic rats inhibits CEL accumulation in lens crystalline, resulting in inhibiting the pathogenesis of cataracts<sup>8</sup>. These reports show the possibility that an inhibiting AGE accumulation is effective in reducing the outbreak of age-related diseases.

In the present study, we evaluated natural materials to prevent AGE formation by utilizing high specific monoclonal antibodies against  $N^{\omega}$ -(carboxymethyl) arginine (CMA), which is reported to accumulate specifically in collagen, and  $N^{\varepsilon}$ -(carboxymethyl)lysine (CML), which is used as an glycative stress marker.

## Materials and Methods Materials

Gelatin (from porcine skin Type A) was purchased from Sigma-Aldrich (St. Louis, MO, USA). D-(-)-ribose was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Immuno Plate (clear flat-bottom immuno non-sterile 96-well plate) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Gelatin hydrolysate was purchased from Sigma-Aldrich. Washing buffer was obtained by the mixture of PBS and Tween 20; a solution containing 0.5% Tween 20 in PBS. Tween 20 (polyoxyethylene (20) sorbitan monoaurate), Sulfuric acid and OPD tablet (1, 2-Phenylenediamine dihydrochloride 5 mg/tablet) was purchased from Wako (Osaka, Japan). Hydrogen peroxide waspurchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG ( $\gamma$ ) was purchased from KPL (Gaithersburg, MD, USA).

## Preparation of natural product samples

Chipped materials were weighed for 10-20 g and divided into three groups. Each group of materials was added 20% of ethanol (EtOH), 50 ml (Nacalai Tesque, Kyoto, Japan), EtOH, 50 ml (Nacalai Tesque) or Methanol (MeOH), 50 ml (Wako), and Acetone, 50 ml (Wako), respectively, and kept still at room temperature for three days. Materials were filtered with a paper filter and concentrated by an evaporator, dried by a freeze dryer to obtain crude-extracted powders.

Each crude extract was dissolved in dimethyl sulfoxide (DMSO, Wako) or H<sub>2</sub>O, and adjusted to 10 mg/mL, 5 mg/mL and 1 mg/mL. Then, the extracts were dissolved in 0.2 M phosphate buffer, mixed with equal volume of ribose (60 mM) and gelatin (4 mg/mL) solution in a clean bench, and sterilized. The ribose-gelatin solution was dispensed into

each tube. Each crude extract sample adjusted to  $100 \ \mu g/mL$ ,  $50 \ \mu g/mL$ , and  $10 \ \mu g/mL$  for the final concentration. Each sample was stirred and incubated at  $37^{\circ}C$  for a week. At the same time, control ribose-gelatin sample without natural products was also incubated at  $37^{\circ}C$  for a week. Furthermore, ribose-gelatin solution was frozen without incubation and used as a negative control.

## Measurement by Enzyme-Linked Immuno-Sorbent Assay (ELISA)

This ELISA was conducted based on Sugawa H *et.al*<sup>9</sup>. Briefly, each well of a 96-well microtiter plate was coated with 100  $\mu$ L of test sample in phosphate-buffered saline (PBS) at the indicated concentration of protein and incubated for 2 h. The wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were then blocked with 0.5% gelatin in PBS for 1 h. After three washes, the wells were incubated for 1 h with 100  $\mu$ L of monoclonal anti-CMA antibody (1  $\mu$ g/mL) or anti-CML antibody (0.5  $\mu$ g/mL). The wells were washed three times and incubated for each 1h with HRP-conjugated anti-mouse IgG followed by 1,2-Phenylenediamine dihydrochloride. The reaction was terminated with 100  $\mu$ L of 1.0 M sulfuric acid, and the absorbance was measured at 492 nm using a micro-ELISA plate reader.

Calculation of inhibition ratio

The absorbance value obtained by the ELISA method was assigned to the following formula and the inhibition rate was calculated.

100 - { (Sample-blank)/(control-blank) ×100}

## Results

The plants used in this study were a total number of 21 plants from the following 9 plant families (the family Lamiaceae, Rutaceae, Compositae, Cruciferae, Saxifragaceae, Umbelliferae, Alliaceae, Zingiberaceae and Aizoaceae). The inhibitory effect of CMA and CML formation was observed in 4 plants, 2 plant families (*Saxifraga stolonifera* of the family Saxifragaceae and *Perillafrutescens* var. crispa, Ocimum basilicum and Glechoma hederacea subsp. grandis of the family Lamiaceae) in this screening.

# *The inhibitory effect of Lamiaceae family on AGE formation*

The inhibition of the CMA formation by using the extract of *Perillafrutescens* var. *crispa* showed at the addition, a concentration of 10 µg/mL, 82.5% in 20% EtOH, 46% in EtOH and 21.7% in acetone. The inhibition of CMA by *Ocimumbasilicum* showed, at 10 µg/mL, 88.5% in 20% EtOH, 18.7% in EtOH and 74.5% in acetone. The inhibition by *Glechoma hederacea* subsp. *grandis* showed, at 10 µg/mL, 11.1% in EtOH. The extracted samples of 20% EtOH tend to have strong inhibitory effects of CMA formation. The *Perillafrutescens* var. crispa of 20% EtOH extract had the inhibition of 82.5% and the *Ocimum basilicum* of 20% EtOH extract had the inhibition of 88.5% (*Fig. 1-A*).

# *The inhibitory effect of Rutaceae family on AGE formation*

The addition of juice sack of Citrus maxima (100 µg/mL) showed the inhibitory effect of CML formation by 45.7% in 20% EtOH extract, 29.9% in EtOH extract and 34% in acetone extract (Fig. 2-B). The juice sack of Citrus limon showed 48.8% in 20% EtOH extract, 35.8% in EtOH extract and 25.8% in acetone extract (Fig. 2-B). The 100 µg/mL of juice sack of Citrus grandis showed 42.5% inhibition of CML formation in 20% EtOH extract, 33.9% in EtOH extract and 33.3% in acetone extract (Fig. 3-B). The inhibition rate of CML formation by juice sack of Citrus reticulata (100 µg/ mL) was 29.7% in 20% EtOH extract, 15.6% in EtOH and 19.6% in acetone extract (Fig. 3-B). In comparison with CMA, the formation of CML was more effectively inhibited by those extracts. The juice sack of Citrus limon was the most effective inhibitor of the samples. The juice sack of Citrus limon, in 20% EtOH extract sample, showed 48.8% inhibition of CML formation at 100 µg/mL and 42.7% inhibition of CML formation at 50 µg/mL (*Fig. 2-B*).

# *The inhibitory effect of Compositae family on AGE formation*

No inhibitory effect on the CMA formation was observed in *Gynura bicolor* at the concentration of 100 µg/mL in 20% EtOH extract (*Fig. 4-A*). *Glebionis coronaria* showed 86.4% in 20% EtOH extract, 91.9% in EtOH extract and 117.5% in acetone extract at the concentration of 100 µg/mL (*Fig. 4-A*). The acetone extract of *Glebionis coronaria* had a strong inhibitory effect. *Glebionis coronaria* showed a tendency that the inhibitory effect was enhanced by decreasing the polarity of extraction solvent (*Fig. 4-A*, *B*).

# The inhibitory effect of Cruciferae family on AGE formation

Inhibitions of CMA formation by Brassica oleracea var. italica, at the concentration of 100 µg/mL, were 46.1% in 20% EtOH extract, 44.4% in EtOH and 20.7% in acetone extract (Fig. 5-A). Inhibitions by Brassica juncea, at the concentration of 100 µg/mL, were 32.2% in 20% EtOH extract, 22.2% in EtOH and 24.5% in acetone extract (Fig. 5-A). There was no sample to possess a strong inhibitory effect. As the sample that showed the strongest effect, Brassica oleracea var. italica showed around 20% inhibition of the CMA formation at all the extraction samples by addition of 10 µg/mL. Furthermore, inhibitions of CMA by the stem of Brassica rapa L., at the concentration of 100 µg/ mL, were 67.4% in 20% EtOH extract, 65.1% in EtOH and 67.8% in acetone extract (Fig. 6-A). Inhibitions of CMA by the stem of Raphanus sativus, at the concentration of 100 µg/mL, were 30.7% in 20% EtOH extract, 16.5% in EtOH and 4.3% in acetone extract (*Fig. 6-A*).

# The inhibitory effect of Saxifragaceae family, Umbelliferae family, Alliaceae family, Zingiberaceae family and Aizoaceae family on AGE formation

Saxifraga stolonifera showed strong inhibitory effects on the formation of CMA and CML. Especially, Saxifraga stolonifera inhibited the CML formation in dose dependent manner (*Fig. 7-B*). The inhibition of the CMA formation by *Zingiber officinale* was, at the concentration of 50  $\mu$ g/mL, 55.7% in 20% EtOH extract (*Fig. 7-A*).

Our present study demonstrated that all the samples of the Lamiaceae family showed inhibitory effects of AGE formation (*Fig. 1*). The strongest inhibitory effect was shown in *Saxifraga stolonifera* and it remains strong inhibition of CMA formation at the concentration of 10 µg/mL (*Fig. 7-A*). Although the samples that were obtained from several parts of Rutaceae family and the Cruciferae family, those samples showed no inhibitory effect (*Fig. 2, 3, 6*).

# Discussion

It has been reported that AGEs are formed from Amadori rearrangement products, which are products in the initial stage of Maillard reaction. The formation of AGEs, in the late stage, involves oxidization and dehydration reactions. Accumulation of AGEs could be accelerated by the development of aging-related diseases including diabetes and arteriosclerosis<sup>10</sup>. Therefore, these diseases are expected to be prevented by inhibiting AGE formation.

*Perillafrutescens* var. *crispa*, *Ocimum basilicum* and *Glechoma hederacea* subsp. *grandis* of the family Lamiaceae, which were recognized in this study to have strong inhibitory effects on the formation of CMA and CML, contain rosmarinic acid and luteolin, and have high antioxidant activities<sup>11</sup>). Saxifraga *stolonifera* contains bergenin, quercetin and gallic acid<sup>12</sup>).

It has also been reported that luteolin inhibited AGE formation through methylglyoxal<sup>13</sup>. Furthermore, a previous study reported that gallic acid was effective to inhibit the CML formation<sup>14</sup>.

Measurements conducted in this study confirmed that natural products that contain these effective chemical compounds had the inhibitory effects on the formation of CMA and CML. Therefore, it was verified that the screening methods reported in this study were simple and instructive to seek effective natural products.

Plants of the family Rutaceae did not show the inhibitory effect on AGE formation, though the plants of the family Rutaceae abundantly contain limonene, which are reported to have ant-inflammatory effects <sup>15)</sup> and antioxidant activities <sup>16)</sup>. Therefore, although several AGEs such as CML is required oxidation process, the plants of the family Rutaceae showed no results for inhibition on AGE formation. Furthermore, *Allium sativum* of the family Alliaceae was recognized to have no inhibitory effect on AGE formation. These results would suggest that allysine <sup>17)</sup>, which are reported to have anti-oxidant activities, however, little inhibitory effect on AGEs. This would suggest that not all of the anti-oxidants are effective on the formation of oxidation-derived AGEs.

The next challenge in the screening of this study is to examine the natural products recognized as inhibitors of the formation of CMA and CML to *in vivo* and clinical trial. Thereby reducing, it would be clarified that these natural products would be beneficial to in our body.



### Fig. 1. Inhibitory effects of the family Lamiaceae on the formation of CMA and CML.

*Perillafrutescens* var. *crispa*, *Ocimumbasilicum* and *Glechoma hederacea* sp. *grandis* inhibit the formation of AGEs. A: inhibitory effect on CMA formation. B: inhibitory effect on CML formation. Ribose-gelatin solutions, with 100  $\mu$ g/mL, 50  $\mu$ g/mL and 10  $\mu$ g/mL of each natural product extract, were incubated for one week. ELISA was performed for the measurement of inhibitory effects (n = 1). AGEs, advanced glycation end products; CMA, N<sup> $\omega$ </sup>-(carboxymethyl)arginine; CML, N<sup> $\varepsilon$ </sup>-(carboxymethyl)lysine; EtOH, ethanol; MeOH, methanol; ELISA, enzyme-linked immuno-sorbent assay.



### Fig. 2. Inhibitory effects of the family Rutaceae-1 on the formation of CMA and CML.

*Citrus maxima* and *Citrus limon* inhibit the formation of AGEs. A: inhibitory effect on CMA formation. B: inhibitory effect on CML formation. Ribose-gelatin solutions, with 100 µg/mL, 50 µg/mL and 10 µg/mL of each natural product extract, were incubated for one week. ELISA was performed for the measurement of inhibitory effects (n=1). AGEs, advanced glycation end products; CMA,  $N^{\omega}$ -(carboxymethyl) arginine; CML,  $N^{\varepsilon}$ -(carboxymethyl)lysine; EtOH, ethanol; ELISA, enzyme-linked immuno-sorbent assay.



Fig. 3. Inhibitory effects of the family Rutaceae-2 on the formation of CMA and CML.

*Citrus grandis* and *Citrus reticulate* inhibit the formation of AGEs. A: inhibitory effect on CMA formation. B: inhibitory effect on CML formation. Ribose-gelatin solutions, with 100 µg/mL, 50 µg/mL and 10 µg/mL of each natural product extract, were incubated for one week. ELISA was performed for the measurement of inhibitory effects (n=1). AGEs, advanced glycation end products; CMA,  $N^{\omega_{-}}$  (carboxymethyl) arginine; CML,  $N^{\varepsilon_{-}}$  (carboxymethyl)lysine; EtOH, ethanol; ELISA, enzyme-linked immuno-sorbent assay.



### Fig. 4. Inhibitory effects of the family Compositae on the formation of CMA and CML.

*Gynura bicolor* and *Glebionis coronaria* inhibit the formation of AGEs. **A:** inhibitory effect on CMA formation. **B:** inhibitory effect on CML formation. Ribose-gelatin solutions, with 100  $\mu$ g/mL, 50  $\mu$ g/mL and 10  $\mu$ g/mL of each natural product extract, were incubated for one week. ELISA was performed for the measurement of inhibitory effects (n = 1). AGEs, advanced glycation end products; CMA,  $N^{\omega}$ -(carboxymethyl)arginine; CML,  $N^{\varepsilon}$ -(carboxymethyl)lysine; EtOH, ethanol; ELISA, enzyme-linked immuno-sorbent assay.



#### Fig. 5. Inhibitory effects of the family Cruciferae-1 on the formation of CMA and CML.

Brassica oleracea var. italica and Brassica juncea inhibit the formation of AGEs. A: inhibitory effect on CMA formation. B: inhibitory effect on CML formation. Ribose-gelatin solutions, with 100  $\mu$ g/mL, 50  $\mu$ g/mL and 10  $\mu$ g/mL of each natural product extract, were incubated for one week. ELISA was performed for the measurement of inhibitory effects (n = 1). AGEs, advanced glycation end products; CMA, N<sup> $\omega$ </sup>-(carboxymethyl)arginine; CML, N<sup> $\varepsilon$ </sup>-(carboxymethyl)ysine; EtOH, ethanol; ELISA, enzyme-linked immuno-sorbent assay.



### Fig. 6. Inhibitory effects of the family Cruciferae-2 on the formation of CMA and CML.

Brassica rapa L. and Raphanus sativus inhibit the formation of AGEs. A: inhibitory effect on CMA formation. B: inhibitory effect on CML formation. Ribose-gelatin solutions, with 100  $\mu$ g/mL, 50  $\mu$ g/mL and 10  $\mu$ g/mL of each natural product extract, were incubated for one week. ELISA was performed for the measurement of inhibitory effects (n = 1). AGEs, advanced glycation end products; CMA, N<sup> $\omega$ </sup>-(carboxymethyl)arginine; CML, N<sup> $\varepsilon$ </sup>-(carboxymethyl)lysine; EtOH, ethanol; ELISA, enzyme-linked immuno-sorbent assay.



#### Fig. 7. Inhibitory effects on the formation of CMA and CML.

The family Saxifragaceae, the family Umbelliferae, the family Alliaceae, the family Zingiberaceae, and the family Aizoaceae: Saxifraga stolonifera, Peucedanum japonicum, Allium sativum, Zingiber officinale and Mesembryant hemum crystallinum inhibit the formation of AGEs. A: inhibitory effect on CMA formation. B: inhibitory effect on CML formation. Ribose-gelatin solutions, with 100  $\mu$ g/mL, 50  $\mu$ g/mL and 10  $\mu$ g/mL of each natural product extract, were incubated for one week. ELISA was performed for the measurement of inhibitory effects (n = 1). AGEs, advanced glycation end products; CMA, N<sup> $\omega$ </sup>-(carboxymethyl)arginine; CML, N<sup> $\varepsilon$ </sup>-(carboxymethyl)lysine; EtOH, ethanol; ELISA, enzyme-linked immuno-sorbent assay.

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## Conflict of interest statement

There are no items deemed to be conflicts of interest in this research.

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