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Original article

Antiglycative effect of genipin and crocetin

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Abstract

Accumulation of advanced glycation end products (AGEs) in the body due to glycative stress is a factor in the onset of aging and lifestyle-related diseases. Suppression of glycative stress is called anti-glycation. Anti-glycation includes suppression of postprandial hyperglycemia, suppression of glycation reaction, and decomposition and excretion of AGEs. Since aminoguanidine (AG), a substance having an AGE production inhibitory effect, has side effects, functional foods having a glycation reaction inhibitory activity are desired. In this study, for evaluating the anti-glycation effect of gardenia (*Gardenia jasminoides* Ellis) extract on the skin proteins, the glycation reaction inhibitory activity and AGE cross-linking breaking activity of genipin and crocetin, purified from gardenia, was measured. Keratin, collagen, and elastin were used as model proteins for the glycation reaction. Genipin and crocetin were found to suppress the production of fluorescent AGEs (F-AGEs), pentosidine, N^{ε}-carboxymethyllysine (CML) and 3-deoxyglucosone (3DG). The activity of genipin on F-AGEs was strong with the half maximal inhibitory concentration (IC_{50}) lower than that of crocetin. Its IC_{50} was 2.4 to 31.6 times lower than that of AG, a positive control. The AGE cross-linking breaking activity was 2.8 times stronger with genipin than with crocetin. These findings indicate that application of a preparation containing genipin and crocetin to the skin may improve the skin condition by suppressing the AGE production from skin proteins and by breaking the AGE cross-linking.

***KEY WORDS*:** advanced glycation end products (AGEs), inhibition of AGE formation, AGE-crosslinks breaking ability, gardenia (*Gardenia jasminoides* Ellis)

Introduction

Accumulation of advanced glycation end products (AGEs) in the body due to glycative stress is a factor in the onset of aging and lifestyle-related diseases. Suppression of glycative stress is called anti-glycation. Anti-glycation includes suppression of postprandial hyperglycemia, suppression of glycation reaction, and decomposition and excretion of AGEs [1, 2]. Aminoguanidine (AG) is known as a substance having an AGE production inhibitory effect [3, 4]. However, ingestion of AG has side effects such as anemia, liver damage, and vitamin B6 deficiency, and has not been put into practical use. Meanwhile, tea/herbal tea [5], vegetables/herbs [6], and fruits [7] have been reported to have an inhibitory effect on glycative reaction. AGE production inhibitory ingredients contained in natural products include chamaemeloside (Roman chamomile) [8], luteolin (edible purple chrysanthemum flower) [9], polymethoxy flavonoid (black galangal or black ginger) [10, 11], cyanidin 3-O-galactoside (red water pepper) [12], caffeoyl glucose (cherry blossom) [13], and rhodanthenone B (mangosteen's peel) [14]. Furthermore, *N*-phenacylthiazolium bromide (PTB) has been reported as a substance that may have a decomposing effect on AGEs [15]. PTB cleaves the α-diketone structure, which is a type of cross-linked structure of AGE-modified proteins [16]. Similar effects have been observed with rosmarinic acid [17], ellagitannin [18], and flavonoids [19].

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Gardenia (*Gardenia jasminoides* Ellis) is a woody plant of the *Rubiaceae* family. Gardenia fruit contains geniposide, a type of iridoid glycoside, and crocetin, a type of carotenoid, and is used as a yellow colorant and as a crude drug for sedation, anti-inflammatory, hemostatic, and antipyretic effects [20]. When geniposide is converted to aglycone by β-glucosidase, it becomes the blue pigment genipin. It has been reported that genipin has a breaking action on the cross-linking of proteins, *i.e.*, collagen, and a suppressing action on the activity of uncoupling protein 2 (UCP2) [21]. Crocetin has been reported to have various functions in vivo, including anti-cancer effect [22], fatigue recovery effect [23], sleep-improving effect [24], and retinal ischemic injury-suppressing effect [25].

In this study, for the purpose of evaluating the anti-glycation effect of the components contained in gardenia on skin proteins, we verified the inhibitory activity on glycation reaction and breaking activity on AGE cross-linking of genipin and crocetin purified from gardenia.

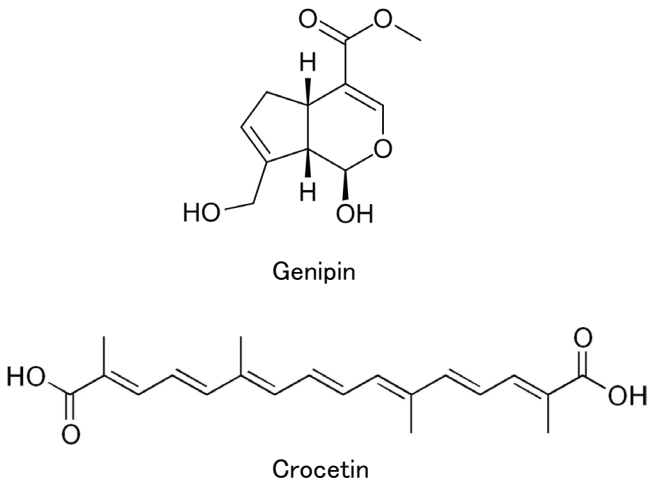

Fig. 1. **Structure of genipin and crocetin.**

Materials and methods

1) Reagent

The reagents used were purchased from the following manufacturers and used; Human serum albumins (HSA, lyophilized powder, ≥ 96%, agarose gel electrophoresis) and *N*-phenacylthiazolium bromide (PTB) are from Sigma-Aldrich Japan (Meguro-ku, Tokyo, Jpam). Collagen Type I (collagen type I, bovine skin, pepsin-solublilized) is from Nippi (Adachi-ku, Tokyo). Elastin peptide (P-Elastin) derived from porcine blood vessels is from Nippon Ham (Osaka, Japan). Aminoguanidine hydrochloride (AG), epigallocatechin gallate (EGCg), 2,3-diaminonaphthalene (DAN), 1-phenyl-1,2-propanedione (1-phenyl)-1,2-propanedione (PPD) and benzoic acid are from Fuji Film Wako Pure Chemical Industries (Osaka). 3-deoxyglucosone (3DG) is from the Dojin Chemical Research Institute (Kamimashiki-gun, Kumamoto, Japan). CircuLex CML/N^{ε}-(Carboxymethyl) Lysine) ELISA Kit is from MBL (Nagoya, Aichi, Japan). Other reagents of special grade or HPLC grade were purchased from Fuji Film Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan).

2) Sample

As samples, a genipin preparation and a crocetin preparation purified from gardenia (Japanese name Kuchinashi) were used (***Fig. 1***). Genipin is formulated with 1,3-butanediol. Crocetin is solubilized by amine compounds and glycerin. These samples were provided by Riken Vitamin (Shinjuku-ku, Tokyo).

3) Protein-glucose glycation model

The protein-glucose glycation model was used for the verification of the glycation reaction inhibitory activity with reference to the previously reported [26]. The composition of the reaction solution was prepared by adding 1/10 amount of the sample solution to 0.1 mol/L phosphate buffer (pH 7.4) containing protein and glucose. The protein and glucose concentrations were as follows; keratin 2.5 mg/mL and glucose 0.6 mol/L, collagen 1.2 mg/mL and glucose 0.4 mol/L, and elastin 6 mg/mL and glucose 0.2 mol/L.

For the glycation reaction, a solution (A) in which all of the phosphate buffer, protein solution, glucose solution, and sample solution are added, a solution (B) in which purified water is added instead of the glucose solution of A, a solution (C) to which purified water was added instead the sample solution of A, and a solution (D) to which purified water was added instead of the glucose solution of C were prepared and incubated at 60 °C for 10 days. The concentrations of AGEs and glycation reaction intermediates in the solution after incubation were measured. AG and EGCg were used as positive controls for the inhibitory activity on glycation reaction.

4) Measurement of AGEs

For fluorescent AGEs (F-AGEs), 200 μL of the glycation reaction solution was placed in a black microplate and the fluorescence derived from AGEs (excitation wavelength 370 nm/fluorescence wavelength 440 nm) was measured according to the previously reported [26]. Pentosidine was measured by HPLC after mixing 50 μL of the reaction solution and 6 N hydrochloric acid and hydrolyzing at 110 °C for 18 hours according to the previously reported [27]. CML was measured using 30 μL of reaction solution using CircuLex CML/N^{ε}-(Carboxymethyl) lysine ELISA Kit.

5) Measurement of glycation reaction intermediates

The glycation reaction intermediate was measured by HPLC after deproteinizing 200 μL of the solution with perchloric acid, adding DAN under alkaline conditions, and labeling according to previously reported [28, 29].

6) Calculation of glycation reaction inhibitory activity

According to the previously reported [26], inhibitory activity on the glycation reaction was calculated by the following formula for the production inhibition ratio (%) of AGEs and glycation reaction intermediates.

The inhibition ratio (%) = {1 – (A – B) / (C – D)} × 100

Furthermore, a half maximal inhibitory concentration (IC_{50} in mg/mL) was calculated from the production inhibition ratio at three concentrations [26, 30]. The smaller the IC_{50} value, the stronger the inhibitory activity.

7) Measurement of AGE cross-linking breaking activity

Evaluation of the AGE cross-linking breaking activity was carried out by mixing the sample solution, 10 mmol/L PPD, and 0.2 mol/L phosphate buffer (pH 7.4) at a ratio of 5: 1: 4 at 37 °C, according to previously reported [15, 18, 19]. After reacting for 8 hours, 0.7 N hydrochloric acid was further added to stop the reaction, and then the amount of benzoic acid cleaved from PPD was measured by HPLC. PTB was used as a positive control for cross-linking breaking activity. When the α-diketone structure of PPD, which is a model substance, is cleaved, 1 mol of benzoic acid is produced from 1 mol of PPD. Using this principle, the cross-linking breaking ratio was calculated by the following formula.

The breaking ratio = {(A – B) / C} × 100

A; Amount of benzoic acid in the reaction solution, B; Amount of benzoic acid in the sample, C; Amount of PPD used in the reaction

Statistical analysis

The measured values are expressed as mean ± standard deviation. Tukey's test or t-test was used to compare the measured values. As a result of statistical analysis, a significance level of less than 5% was considered significant.

Results

Inhibitory activity on F-AGE production in three types of protein-glucose glycation model

The inhibitory activity of genipin and crocetin on F-AGEs production was found to be concentration-dependent in all three reaction models using keratin, collagen, and elastin as model proteins (***Fig. 2***). The inhibitory activity on F-AGEs production at a genipin concentration of 0.4 mg/mL was stronger in the order of elastin (inhibition ratio: 96.9%), keratin (83.6%), and collagen (71.1%). Similarly, the activity of crocetin was stronger in the order of keratin (80.1%), collagen (61.5%), and elastin (12.2%). The F-AGE production inhibition ratio of genipin and crocetin were 1.0-fold different for keratin, 1.2-fold for collagen, and 7.9-fold for elastin. IC_{50} values for keratin, collagen, and elastin were lower for genipin than for crocetin (***Table 1***). The IC_{50} values of genipin and crocetin were lower than those of AG in all combinations except the IC_{50} value of crocetin for elastin.

Inhibitory activity on production of AGEs and intermediates in keratin-glucose glycation model

Inhibitory activity of 0.4 mg/mL genipin and 0.4 mg/mL crocetin was examined on production of F-AGEs, pentosidine, CML, and 3DG in the keratin-glucose glycation model (***Table 2***). The inhibitory activity of genipin was stronger than that of crocetin in all the verified items. In particular, the inhibition ratio of pentosidine production (97.1%) of genipin was 2.1 times that of crocetin.

Breaking activity on AGE cross-linking

The AGE cross-linking breaking activity of genipin (18.6%) was 2.8 times stronger than crocetin (6.7%, ***Fig. 3***). The breaking ratio of 2 mg/mL genipin was 0.7 times that of 5 mmol/mL PTB (breaking ratio 25.5%), a positive control.

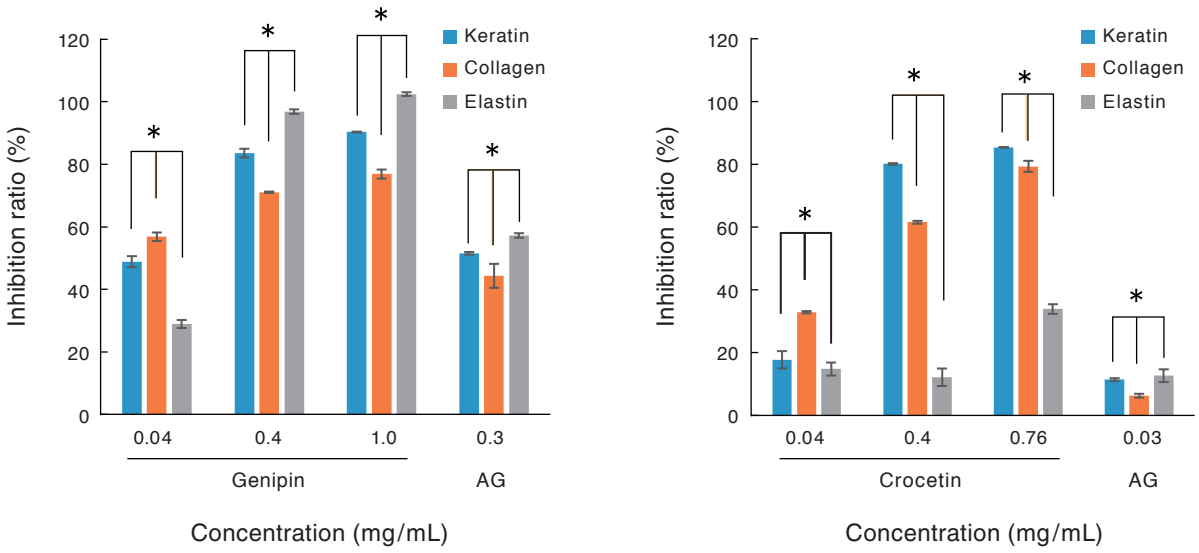

Fig. 2. **Inhibitory activity of genipin and crocetin on F-AGEs formation.**

Results are expressed as mean ± SD, n = 3; * $p < 0.05$ by Tukey's test. AG, aminoguanidine; F-AGEs, fluorescent advanced glycation end products; SD, standard deviation.

Table 1. IC_{50} on the protein-glucose glycation model.

Model protein	Genipin (mg/mL)	Crocetin (mg/mL)	Aminoguanidine [1] (mg/mL)
Keratin	0.041	0.145	0.275
Collagen	0.013	0.135	0.423
Elastin	0.086	1.0 >	0.207

1) Positive control of glycation inhibitor. IC_{50}, Half maximal inhibitory concentration.

Table 2. AGE inhibition ratio on the keratin-glucose glycation model.

AGE compound	Genipin [1] (%)	Crocetin [1] (%)
Fluorescent AGEs [2]	83.6 ± 0.1	80.1 ± 0.05
Pentosidine	97.1 ± 0.5	45.4 ± 5.5
CML [3]	71.0 ± 1.8	50.5 ± 0.3
3DG [4]	46.9 ± 0.2	27.9 ± 0.1

1) Sample concentration: 0.4 mg/mL, mean ± SD (n = 3). 2) Characteristic fluorescence (excitation/emission = 370/440 nm). 3) N^{ε}-carboxymethyl lysine. 4) 3-deoxyglucosone. AGEs, advanced glycation end products; SD, standard deviation.

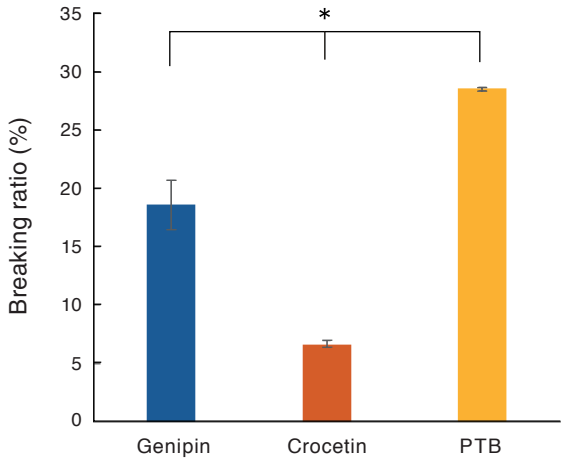

***Fig. 3.* AGE cross-link breaking activity.**

Results are expressed as mean ± SD, n = 3; * $p < 0.05$ by Tukey' s test. PTB, 5 mmol/L N-phenacylthiazolium bromide; AGE, advanced glycation end product; SD, standard deviation.

Discussion

Inhibitory activity of genipin and crocetin on glycation reaction

Iridoid glycosides, *i.e.* genipin, are contained in many medicinal plants other than gardenia[31]. Noni (*Morinda citrifolia* L.) fruits contain iridoids such as deacetylasperulosidic acid and loganic acid, and have been reported to have an AGE production inhibitory action[32]. Persons who take noni fruit juice daily have less accumulation of AGEs in their skin[33]. In addition, carotenoids, *i.e.* crocetin, have been reported to be useful for anti-oxidant, anti-inflammatory, and ocular disease protection[34]. The anti-glycation effect of carotenoids has been reported for β-carotene[35] and astaxanthin[36]. Crocin, which has two molecular bindings of crocetin, has been verified to have an inhibitory effect on AGE production on crystallin in the rat eye lens[37]. Also, the IC_{50} value of the AGE production inhibitory activity of silibinin, a flavonolignan, was 0.0702 mg/mL, which was 4.8 times lower than that of AG, a positive control[38]. A gel formulation of 1% milk thistle (*Silybum marianum* (L.) Gaertn) flower extract, which contains 0.24% silibinin, prevented the decrease in network formation of fibulin-1 and reduced CML expression in human skin explant model treated with methylglyoxal. The anti-glycation effect of silibinin is thought to be involved in the suppression of protein carbonylation. A similar effect has been observed in akebi (*Akebia quinata* Decaisne) fruit extract (AQFE), which has the same level of inhibitory activity on the F-AGE production as AG[39].

In this study, we examined the AGE production inhibitory activity using skin protein, *i.e.* keratin, collagen, and elastin, as model proteins for the purpose of verifying the anti-glycation effect of genipin and crocetin contained in gardenia. As a result, genipin and crocetin were found to suppress the production of F-AGEs, pentosidine, CML, and 3DG in all three types of glycation reaction models. These effects were stronger with genipin than with crocetin. A strong inhibitory activity of genipin was observed and its IC_{50} of F-AGEs in the three models was 0.013 to 0.086 mg/mL, which was 2.4 to 31.6 times lower than that of AG. AG suppresses the AGE formation by blocking glycation reaction intermediates with carbonyl groups[3]. Combined with these facts, skin application of a gel containing genipin and crocetin may suppress the AGE production in the skin by blocking the carbonyl group of the intermediates and by anti-oxidative action as in silibinin and AQFE.

Breaking activity on AGE cross-linking of genipin and crocetin

Genipin and crocetin were found to have an AGE cross-linking breaking activity. The PTB, a positive control in this study, cleaves the dicarbonyl bond in the PPD molecule to produce benzoic acid, indicating the possibility of cleaving the dicarbonyl structure formed by glycation cross-linking of proteins[15]. It has also been reported that PTB reduces AGEs in rat arterial walls[40]. However, another study showed that PTB did not reduce in AGE cross-linking of tail collagen in rats[41]. Further verification is required for the effect of degrading AGEs in human tissues by ingestion of AGE cross-linking breaking substances. On the other hand, the AGE decomposing effect of the cross-linking breaking component by skin application has been reported in the extracts of mugwort (YAC extract) and *Astragalus sinicus* L[42, 43]. In a study in which a solution containing ethanol extract of mugwort (1% YAC extract) was added and incubated dropwise to a plate coated with AGE-formed collagen gel, the AGEs on the collagen gel surface almost disappeared. In a clinical study in which 38 healthy middle-aged women used a skin care product containing 1% YAC extract for 6 months, the elasticity reduction index (RRT values) and sagging index (Sagging index) in the central part of the left cheek improved. Since genipin and crocetin have an AGE cross-linking breaking activity like YAC extract, skin application of these components may lead to reduction of skin AGEs and improvement of skin condition.

Conclusion

For the purpose of verifying the anti-glycation effect of genipin and crocetin contained in gardenia on skin proteins, inhibitory activity on AGE production and breaking activity on AGEs cross-linking were verified in the glycation model using keratin, collagen, and elastin as model proteins. Genipin and crocetin were found to have inhibitory actions on production of F-AGEs, pentosidine, CML, and 3DG. These findings indicate that topical application of a preparation containing genipin and crocetin may improve the skin condition by suppressing the AGE production and by breaking the AGE cross-linking.

Conflict of interest declaration

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