

Original article

In Vitro Effects of *Geranium dielsianum* Extract on Glycative Stress

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Abstract

Objective: *Geranium dielsianum* (GD) has long been recognized as being pharmacologically effective; however, its biological effects remain to be clarified in full. This study was conducted to evaluate the functionality of GD from an anti-glycative viewpoint, so as to obtain basic data on the potential of GD extract for reducing glycative stress.

Methods: Sucrase and maltase inhibition activities were evaluated as markers of the α -glucosidase inhibition activity of GD extract. The suppression of *N*^ε-(carboxymethyl) lysine (CML) production was analyzed using ELISA in a glucose/type 1 collagen *in vitro* reaction system. Oxygen radical absorbance capacity (ORAC) was evaluated as an index of antioxidant capacity.

Results: The sucrase inhibition activity of GD was determined to be 99.7% for freeze-dried extract, and 93.0% for MISKAMISKA[®], a GD extract product [GDE], with an IC₅₀ of 0.027 mg/mL for freeze-dried extract, and 0.028 mg/mL for GDE. The maltase inhibition activity of GDE was determined to be 96.0%, with an IC₅₀ of 0.016 mg/mL. The IC₅₀ of GDE for CML suppression activity (Col.) was calculated as 0.0065 mg/mL, demonstrating that GDE is 24.6 times more potent than the positive control, aminoguanidine. The ORAC of GDE was determined to be 5.1×10^3 μ mol/g.

Conclusion: The present study showed that GDE has inhibitory effects on postprandial hyperglycemia, as well as anti-oxidative effects, and anti-glycation effects *in vitro*, suggesting its potential for use as a food that reduces glycative stress.

KEY WORDS: *Geranium dielsianum*, advanced glycation end products (AGEs), oxygen radical absorbance capacity (ORAC), α -glucosidase, *N*^ε-(carboxymethyl)lysine (CML)

Introduction

Geranium dielsianum (GD), a perennial herbaceous dicotyledonous plant belonging to the family *Geraniaceae*, is reportedly indigenous exclusively to the high lands of altitudes of 3,500 m or higher in the Central Andean Mountains in Peru; it has long been recognized as being pharmacologically effective against diabetes, throat inflammation, and aphtha, as well as for blood purification and other effects¹⁾. In Peru, GD is infused to make a popular herbal tea. An extract of GD, which is commercially available under the tradename MISKAMISKA[®], has been reported to improve the intestinal environment^{2,3)}.

GD extracts were reported to have inhibitory effects on α -glucosidase⁴⁾, an enzyme that catalyzes the hydrolytic reaction of α -glycoside bonds in sugars. Suppression of this enzyme function decreases glucose absorption from the small

intestine into the blood.

At elevated blood glucose levels, reducing sugars (such as glucose and fructose) and proteins bind together non-enzymatically to promote the production of glycoproteins (glycation reaction). As the glycation reaction proceeds, advanced glycation end products (AGEs) form and deposit on tissue^{5,6)}, and then bind to the Receptor for AGEs (RAGE) and other receptors⁷⁾, and consequently inducing disorders in a wide variety of organs and tissues⁸⁾. The concept of glycative stress involves these comprehensive aspects⁹⁾. Therefore, GD extracts that suppress blood glucose level elevations are likely to reduce glycative stress. Against this background, the present study was conducted to evaluate the functionality of GD from an anti-glycative viewpoint, so as to obtain basic data on the potential of its extract for reducing glycative stress.

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Methods

Sample preparation

A flowchart of the study is shown in [Fig. 1](#).

As test samples, 22 GD-containing plant materials from Peru were used. After milling each sample, 60% ethanol was added, and the mixture was extracted at room temperature. The liquid extract was aspiration-filtered, and the resulting filtrate was concentrated under reduced pressure using an evaporator, and then freeze-dried (FD) to yield an experimental sample. A hydrated-ethanol extract of GD (GDE; product name, MISKAMISKA[®], manufactured by TOWA CORPORATION Ltd., Tokyo, Japan) was also used. Acarbose powder (Bayer Yakuhin, Osaka, Japan), a component in the antidiabetic therapeutic tablet, Glucobay (Bayer Yakuhin), served as the positive control.

α -Glucosidase inhibition activity

Alpha-glucosidase inhibition activity was determined as follows: A mucosa sample was collected from each male Wistar rat (weighing 300 to 400 g), and prepared as a crude enzyme fraction. After adding 1.0 mL of the Glucose CII-Test Wako coloring reagent (Wako Pure Chemical Industries, Osaka, Japan), the two components were mixed thoroughly, and the reaction was carried out at 37°C for 7 minutes. The resulting glucose was quantified using a spectrophotometer (U-2900; Hitachi High-Technologies Corporation, Tokyo, Japan) at a wavelength of 505 nm.

Determination of protein content

Protein was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan). Each sample was treated with α -glucosidase to yield a crude enzymolysis fraction (precipitate), which was dissolved in 500 μ L of 0.1 M maleate buffer solution, and diluted 20 fold with distilled water. BCA Protein Assay Reagents A and B were mixed in a 50:1 ratio, and the color former was added at 1 mL per sample, after which the mixture was incubated at 37°C for 30 minutes. After returning to room temperature, the mixture was tested using a spectrophotometer at 562 nm. A working curve was generated using solutions of Albumin Standard (bovine serum albumin: BSA) in distilled water at concentrations of 500, 250, 125, 62.5, 31.25, and 0 mg/mL.

AGE production inhibition activity

● CML production inhibition

The activity to suppress the production of *N*^ε-(carboxymethyl) lysine (CML), an AGE, was measured using the following glucose/collagen *in vitro* reaction system: The test material was GDE, with aminoguanidine serving as the positive control¹⁰. Each sample (48.1 mg) was weighed and dissolved in 4.8 mL of distilled water. This 10 mg/mL solution, as the stock solution, was diluted to five different concentrations from 0.05 to 3 mg/mL. Aminoguanidine was prepared in solution to four concentrations from 0.1 to 3 mg/mL. Each prepared sample was added to a reaction mixture of 0.05 mol/L phosphate-buffered solution (pH 7.4), 1.2 mg/mL collagen

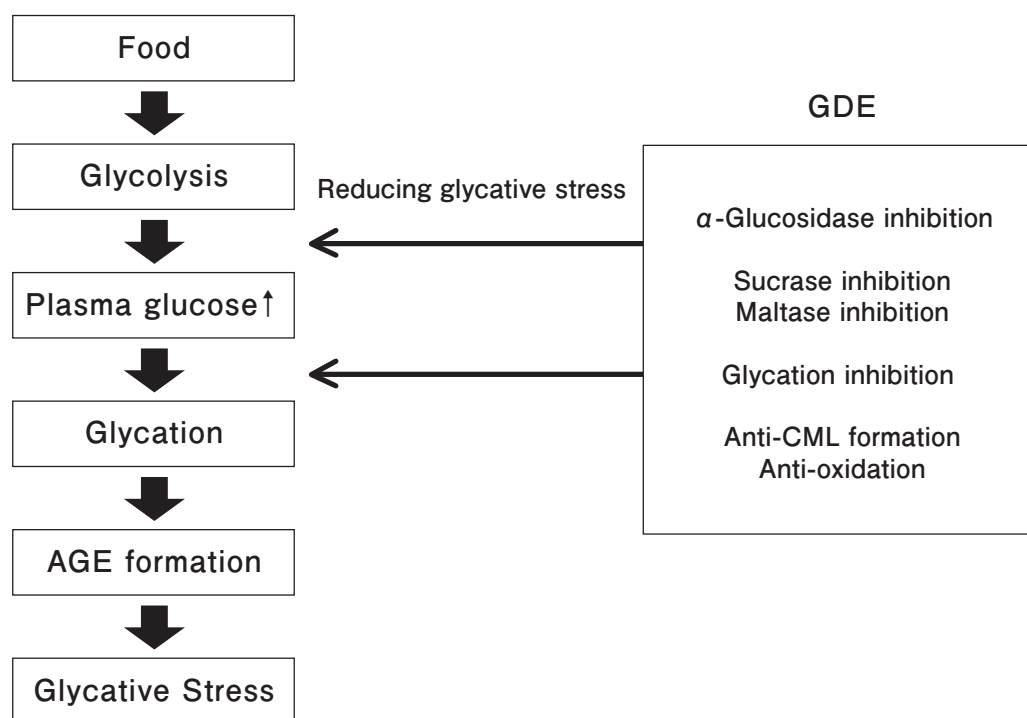


Fig. 1. Experimental flow chart of this study.

GDE, *Geranium dielsianum* extract (MISKAMISKA[®]).

type I (bovine dermal collagen; Nippi, Inc., Tokyo, Japan), and 0.4 mol/L glucose, to obtain a one-tenth concentration, and the mixture was incubated at 60°C for 10 days. Distilled water was added in place of each sample as the negative control. Anti-CML activity was measured using the incubated reaction mixture^{11,12}.

The CML produced in the sample reaction liquid was quantified by ELISA using the CircuLex CML/*N*^ε- (carboxymethyl) lysine ELISA KIT (Cyclex Co., Ltd., Nagano, Japan). As an index of suppression of CML production, IC₅₀ (50% production inhibitory concentration, per solid concentration) was calculated.

Antioxidant activity

To evaluate the antioxidant capability of GDE, oxygen radical absorbance capacity (ORAC) was determined (Japan Food Research Laboratories, Tokyo, Japan)¹³. An extract of each sample with 50% ethanol was added to a liquid mixture of 2,2'-azobis-(2-methylpropionamide) dihydrochloride (AAPH) and the fluorescent substance, fluorescein, and the fluorescence intensity decay time (excitation light wavelength: 485 nm, fluorescence wavelength: 520 nm) was measured over time. On the basis of a graph of the relationship between measuring time and fluorescence intensity, the antioxidant power was evaluated. Measured results were displayed on the basis of the unit activity exhibited by 1 μmol of Trolox.

Results

α-Glucosidase inhibition activity

● Sucrase inhibition

Non-acarbose samples were prepared to a final concentration of 0.1 mg/mL, and screened for sucrase inhibition (Fig. 2). The inhibition rate was 38.0% for acarbose (0.000125 mg/mL), 54.7% for acarbose (0.00025 mg/mL), 69.8% for acarbose (0.0005 mg/mL), 93.0% for GDE (MISKAMISKA®), 99.7% for GD, 30.2% for abuta [*Abuta grandifolia* (Mart.) Sandwith]), 60.5% for achiote [*Bixa orellana* L.], 70.3% for arrayan [*Myrtus communis* L.], 5.9% for artemisa [*Artemisia vulgaris*], 12.7% for calaguala [*Polypodium calaguala*], 35.4% for catuaba [*Erythroxylum catuaba*], 16.8% for canchalagua [*Schkuhria pinnata*], 13.6% for cedron [*Aloysia triphylla*], 23.9% for chuchuhuasi [*Maytenus Macrocarpa*], 27.4% for coffeeberry [*Coffea*], 4.0% for comfrey [*Symphytum officinale* L.], 3.4% for diente de leon [*Taraxacum officinale* Weber], 16.0% for flor blanca [*Buddleja incana*], 18.1% for hierba luisa [*Lippia triphylla*], 50.5% for huamanpinta [*Chuquiraga Spinoza*], 0.4% for huayruro colorado [*Ormosis coccinea* Jacq.], 57.3% for iporuro [*Alchornea castaneifolia*], 20.6% for malva [*Malva sylvestris*], 21.4% for manayupa [*Desmodium molliculum*], and 21.8% for pimpinella [*Pimpinella anisum* L.] (Fig. 2).

The IC₅₀ values of GDE and GD were determined to be 0.028 and 0.027 mg/mL, respectively (Fig. 3). All inhibition rate data were corrected with the 54.7% mean inhibition rate from the measurements obtained in this experiment at a final concentration of 0.00025 mg/mL of the positive control acarbose powder. The IC₅₀ of acarbose was 0.00025 mg/mL (Fig. 4).

● Maltase inhibition

The maltase inhibition activity of each sample was determined to be 96.0% for GDE, 60.5% for acarbose (0.00025 mg/mL), and 35.9% for mulberry leaf extract (Fig. 5). The IC₅₀ was determined to be 0.016 mg/mL for GDE, and 0.0001 mg/mL for acarbose (Figs. 6 and 7).

AGE production inhibition activity

CML production suppression

GDE and aminoguanidine suppressed CML production in a concentration-dependent manner (Figs. 8 and 9). The IC₅₀ of GDE for anti-CML activity (Col.) was determined to be 0.0065 mg/mL, and that of aminoguanidine for CML production suppression activity (Col.) was 0.16 mg/mL.

Antioxidant activity

The ORAC of GDE was determined to be 5.1×10³ μmolTE/g.

Discussion

α-Glucosidase inhibition

In the present study, the α-glucosidase inhibition activities of various GD extracts were evaluated, and their IC₅₀ values were calculated. Alpha-glucosidase is a generic name for a number of enzymes that decompose respective sugars, including maltase, which hydrolyzes maltose into two glucose molecules: galactase, which decomposes lactose into glucose and galactose; and sucrase, which decomposes sucrose into glucose and fructose, on the small intestinal mucosa. In the present study, inhibitory activities against two enzymes (sucrase and maltase) were analyzed. Screening determinations revealed high sucrase inhibition activity in GD and GDE (Fig. 2). In addition, GDE was found to have higher maltase inhibition activity, in terms of IC₅₀, than mulberry leaf extract which is commercially available as a dietary supplement and has been thought to have anti-diabetic effects (Fig. 5). An earlier study showed that methanol extracts of GDE have inhibitory effects on α-glucosidase, hyaluronidase, elastase, and collagenase *in vitro*⁴. The present study showed that hydrated-ethanol extracts of GD also have inhibitory effects on α-glucosidase activity.

A proposed mechanism for glycative stress reduction involves the prevention of postprandial hyperglycemia¹⁴⁻¹⁶ (Fig. 1). Biological proteins react with glucose and other reducing sugars in a non-enzymatic, irreversible manner (glycation reaction), producing advanced glycation end products (AGEs), which are associated with aging and a wide variety of diseases¹⁷. The glycation reaction proceeds further with increasing blood sugar concentration; therefore, a prolonged postprandial hyperglycemic state represents exposure to glycative stress. Postprandial hyperglycemia can reportedly be suppressed by eating grapefruit¹⁴, adding dietary fiber to meals¹⁵, and eating staple food, not alone, but together with side dishes¹⁶.

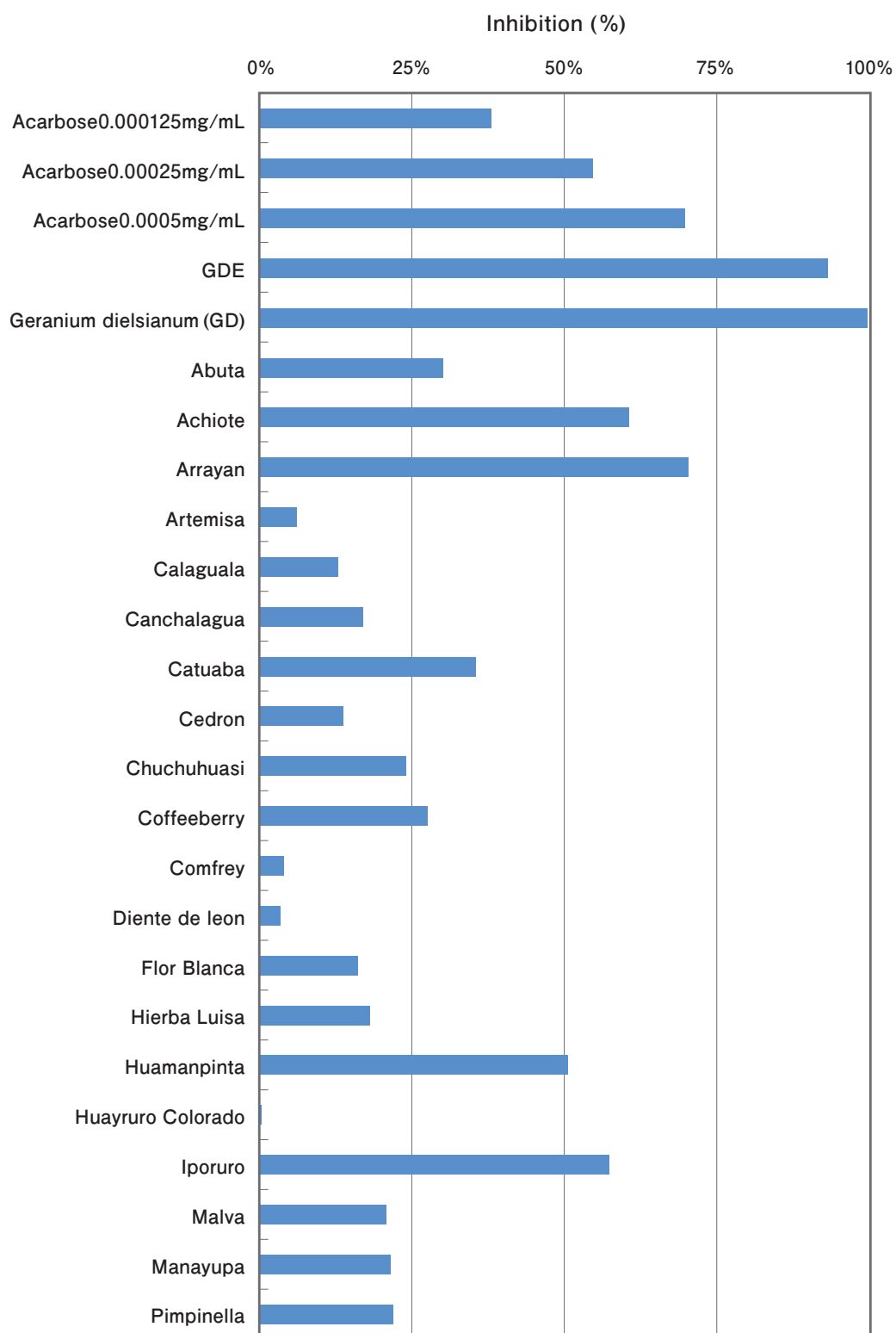


Fig. 2. α -Glucosidase (sucrase) inhibition activity of materials in Peru.

Data are expressed as % inhibition. Test materials are extracted in 60% ethanol and freeze-dried. Measurement time; n = 1. GD, *Geranium dielsianum*; GDE, GD Extract (MISKAMISKA[®]).

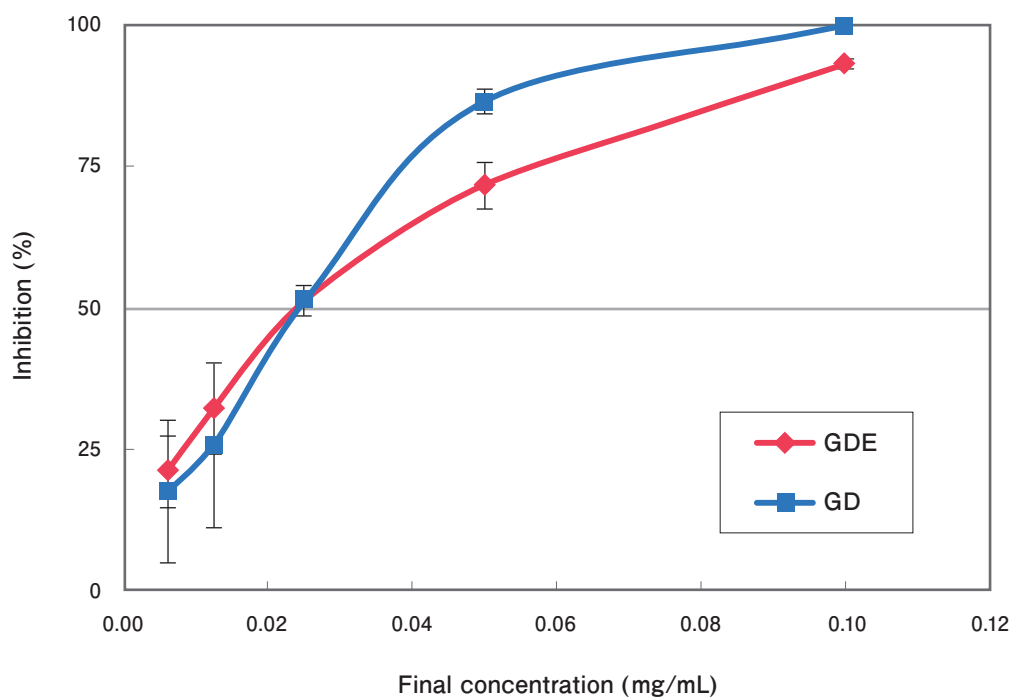


Fig. 3. Sample doses and α -Glucosidase (sucrase) inhibition.

Results are expressed as mean \pm standard deviation of % inhibition of the enzyme activity. IC₅₀; 0.028 mg/mL in GDE, 0.027 mg/mL in GD. Measurement time; n = 3. IC₅₀, 50% inhibition concentration; GD, *Geranium dielsianum*; GDE, GD Extract (MISKAMISKA[®]).

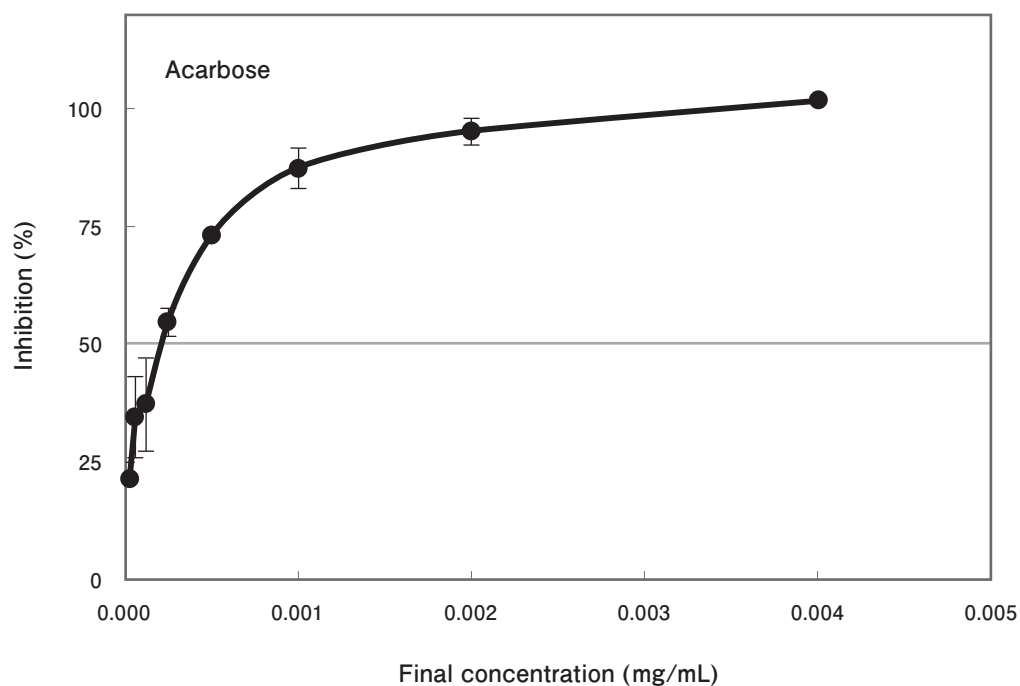


Fig. 4. Sample doses and α -Glucosidase (sucrase) inhibition by Acarbose.

Results are expressed as mean \pm standard deviation of % inhibition of the enzyme activity. IC₅₀, 0.00025 mg/mL in Acarbose. Measurement time; n = 4. IC₅₀, 50% inhibition concentration.

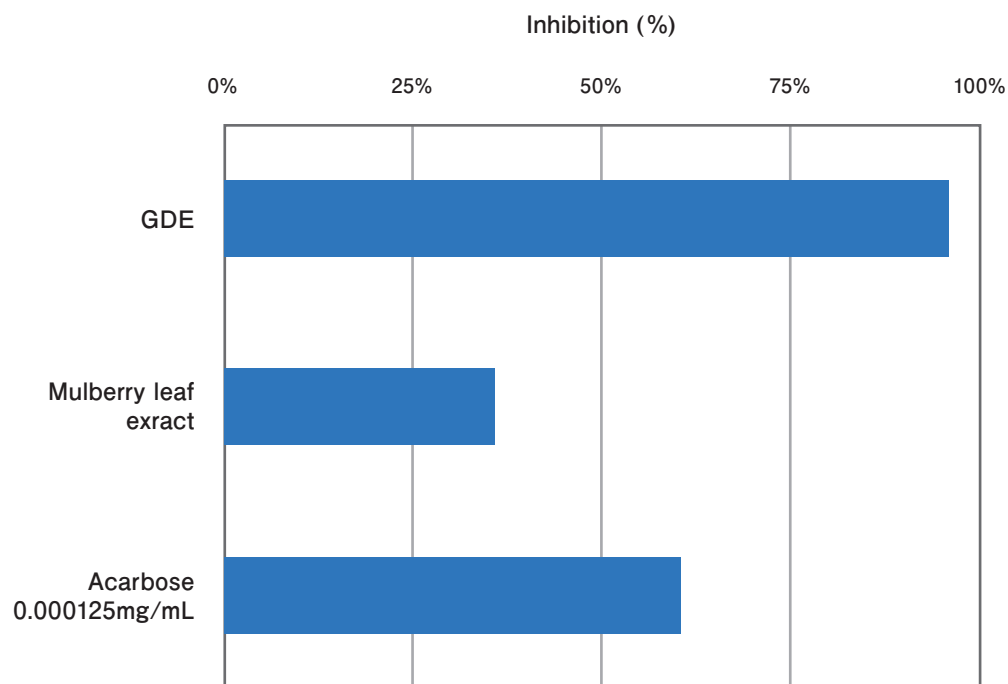


Fig. 5. α -Glucosidase (maltase) inhibition activity of materials.

Data are expressed as % inhibition. Measurement time, n = 1. GDE, *Geranium dielsianum* extract (MISKAMISKA[®]).

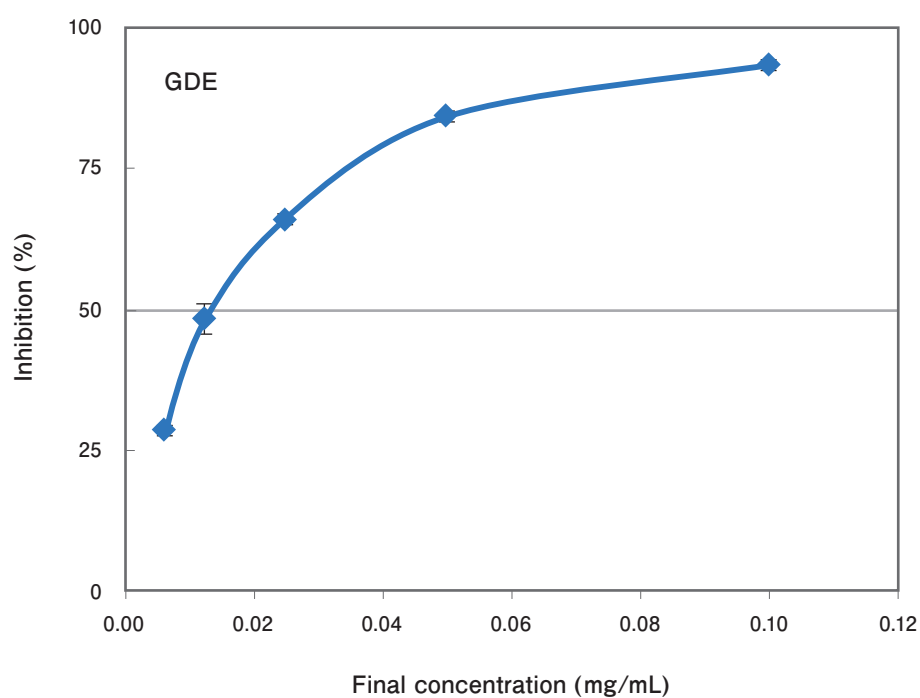


Fig. 6. Sample doses of GDE and α -Glucosidase (maltase) inhibition.

Results are expressed as mean \pm standard deviation of % inhibition of the enzyme activity. Measurement time, n = 3. IC₅₀ = 0.016 mg/mL. IC₅₀, 50% inhibition concentration; GDE, *Geranium dielsianum* extract (MISKAMISKA[®]).

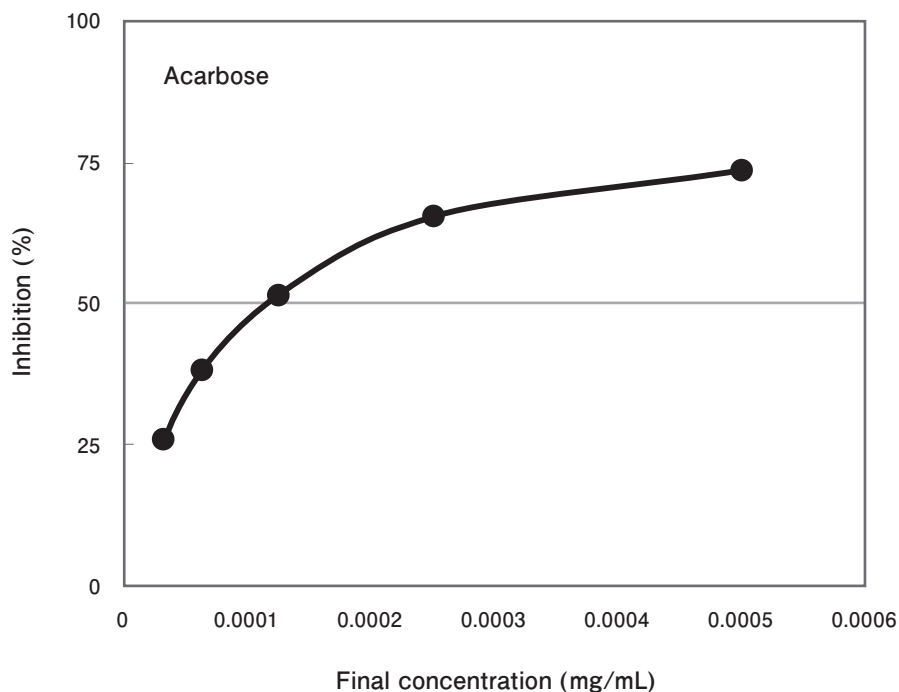


Fig. 7. Sample doses of Acarbose and α -Glucosidase (maltase) inhibition.

Results are expressed as average values of % inhibition of the enzyme activity. Measurement time, n = 2. IC₅₀ = 0.0001 mg/mL. IC₅₀, 50% inhibition concentration.

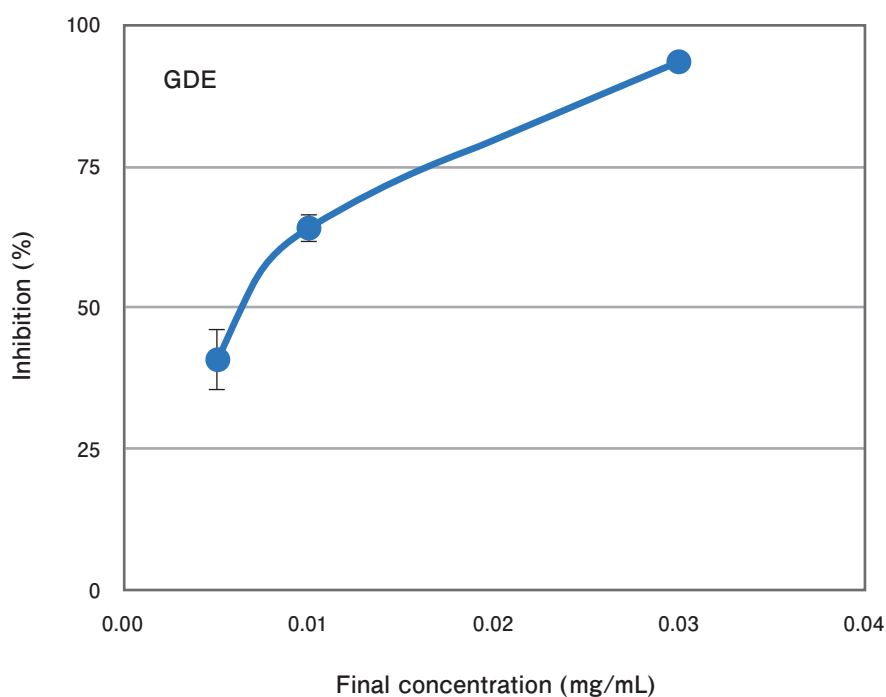


Fig. 8. Effect of GDE on *in vitro* CML formation.

Data are expressed as mean \pm standard deviation of % inhibition of CML formation in *in vitro* reaction between glucose and type 1 collagen. Measurement time, n = 3. IC₅₀ = 0.0065 mg/mL. IC₅₀, 50% inhibition concentration; GDE, *Geranium dielsianum* extract (MISKAMISKA®); CML, N ϵ -(carboxymethyl)lysine.

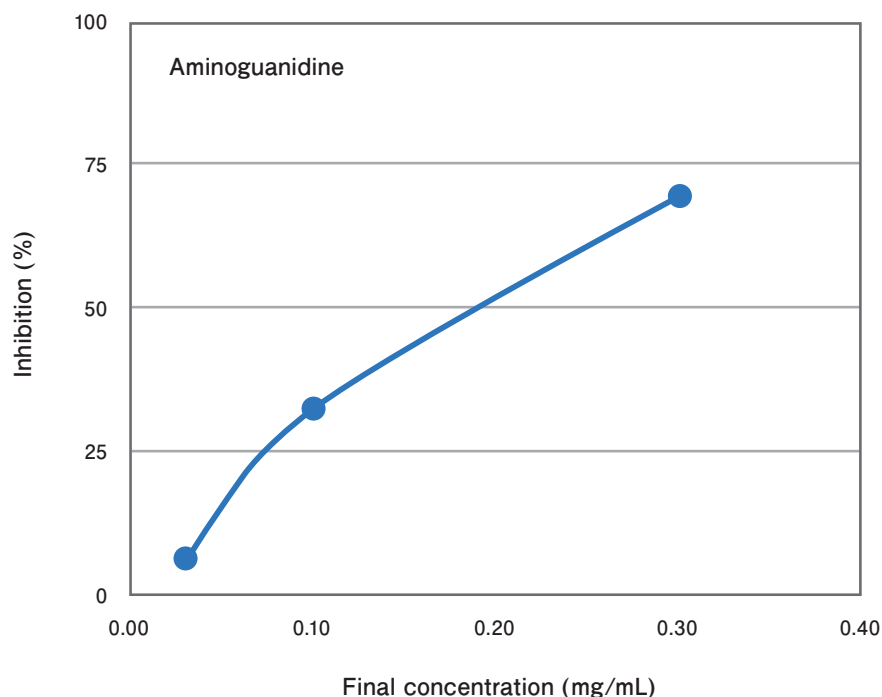


Fig. 9. Effect of aminoguanidine on *in vitro* CML formation.

Data are expressed as average values of % inhibition of CML formation in *in vitro* reaction between glucose and type 1 collagen. Measurement time, n = 2. IC₅₀ = 0.16 mg/mL. IC₅₀, 50% inhibition concentration; N^ε-(carboxymethyl)lysine.

AGE production suppression activity

Earlier studies found anti-glycation activity (AGE production suppression activity) in a wide variety of herbal teas⁶, fruits¹⁸, herbs and vegetables¹⁹, and spices²⁰. In the present study, GD, which possesses α -glucosidase inhibition activity, was examined for suppression of CML production. The IC₅₀ for the α -glucosidase inhibition activity was determined to be 0.0065 mg/mL for GDE, and 0.16 mg/mL for aminoguanidine, demonstrating that GDE is 24.6 times more potent in CML suppression than aminoguanidine (Figs. 7 and 8).

Oxygen radical absorption capacity (ORAC) is an index of antioxidant capacity (reactive oxygen scavenging potential) expressed per gram of vegetable, fruit, and other food samples. The ORAC of GDE is 5.1×10^3 $\mu\text{molTE/g}$, demonstrating a much higher reactive oxygen scavenging potential than that of vegetables and fruits having relatively high ORAC values: prunes (57.7 $\mu\text{molTE/g}$), raisins (28.3 $\mu\text{molTE/g}$), blueberries (24.0 $\mu\text{molTE/g}$), blackberries (20.0 $\mu\text{molTE/g}$), garlic (19.4 $\mu\text{molTE/g}$), kale (17.7 $\mu\text{molTE/g}$), red wine (16.7 $\mu\text{molTE/g}$), and spinach (12.6 $\mu\text{molTE/g}$)²¹. Furthermore, an earlier study reported that hot water extracts of GDE also possess potent antioxidant activity²². These facts suggest that the antioxidant capacity of GDE strengthens the anti-glycation effects.

CML is produced through a reaction of either glyoxal (GO) from oxidative cleavage of an Amadori compound and peroxidation of a lipid, or glycol aldehyde from hypochlorous acid and serine, and lysine residues²³. Its production is promoted in the presence of reactive oxygen species, such as hydroxy radicals ($\bullet\text{OH}$) and superoxynitrites ($\text{ONOO}\bullet$). In addition to CML, numerous AGEs are produced by oxidizing reactions, including pentosidine^{24,25}; therefore, GDE is likely to suppress the production of such AGEs; anti-oxidation helps anti-glycation, resulting in decreased glycative stress.

CML, a representative skin-accumulating AGE, is also found in the epidermal layer, where metabolic turnovers occur relatively rapidly²⁶. The most influential glycative stress on the skin is the accumulation of AGEs⁹. CML is associated with aging and various diseases in many manners, including apoptosis induced in human skin fibroblasts by the addition of CML-modified collagen²⁷. Therefore, with its potent activity to suppress CML production, GDE is expected to serve as a new anti-glycation food.

Conclusion

The present study showed that GDE (MISKAMISKA[®]) has inhibitory effects on postprandial hyperglycemia, anti-oxidative effects, and anti-glycation effects *in vitro*, suggesting its potential for use as a food that reduces glycative stress.

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Conflict of interest

A part of this study was supported by TOWA CORPORATON Ltd.

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