Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : July 6, 2021
Accepted : August 25, 2021
Published online : September 30, 2021
doi:10.24659/gsr.8.3_148

Original article

Screening plant extracts for inhibition of hair glycation

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Abstract

***Objectives*:** It has been reported that hair glycated over time from the root to the tip exhibits changes in physical properties, such as a decrease in breaking strength. However, few studies have examined the inhibition of hair glycation. In this study, we examined the anti-glycation effect of plant extracts to identify materials that inhibit hair glycation.

***Methods*:** A total of 26 plant extracts were used as the cosmetic ingredients. A glycation solution was prepared with 0.1 mol/L phosphate buffer (PB; pH 7.4), 5.0 mol/L glucose, and 5.0 mg/mL keratin; the sample to be tested was then added to this solution. After thermal reaction, the levels of advanced glycation end products (AGEs) produced in the reaction solution were measured using the fluorescence method (excitation wavelength 370 nm / detection wavelength 440 nm), and the AGE production inhibitory rate was calculated. In the primary screening, each sample was adjusted to a final concentration of 0.1 mg/mL in the reaction solution with deionized water, and the AGE production inhibition rate was measured. In the secondary screening, half maximal inhibitory concentration (IC_{50}) was calculated and evaluated for samples with an AGE production inhibition rate of 75% or more obtained in the primary screening. Next, we obtained hair samples from healthy women in their 30s who had no history of beauty treatment, and verified the anti-glycation effects of the plant extracts. To verify the anti-glycation effect, the hair sample was immersed in 0.1 mol/L phosphate buffer (pH 7.4) containing 1.2 mol/L of glucose and 0.1 mg/mL of each sample, and reacted at 50°C for 10 days. Hair protein was extracted, and the levels of fluorescent AGEs were measured. The physical properties of the hair were measured using a tensile tester at ambient temperature (25°C) and humidity (65% RH).

***Results*:** Following primary screening, 75% or more of the AGE production inhibitory effect was observed in 9 out of 26 samples. The secondary screening results showed that the IC_{50} of rooibos extract had a stronger anti-glycation effect than the IC_{50} of aminoguanidine. An *in vitro* hair test showed that rooibos extract inhibited the production of fluorescent AGEs (production inhibition rate 82.4%). In addition, the breaking strength of the glycated hair was reduced by 20.8% compared to that of the un-glycated hair; however, after treatment with the rooibos extract, the reduction in breaking strength due to glycation could be suppressed to 4.9%.

***Conclusion*:** Rooibos extract was found to have an inhibitory effect on the production of fluorescent AGEs in hair proteins. It also showed an inhibitory effect on the decrease in the breaking strength of the hair due to glycation. These results indicate that the addition of rooibos extract to hair cosmetics may help inhibit the changes in the physical properties of hair caused by glycation.

***KEY WORDS*:** hair, plant extract, Rooibos, glycation, advanced glycation endproducts (AGEs)

Introduction

The non-enzymatic reaction between reducing sugars, such as glucose and proteins, is called glycation. Glycation also occurs *in vivo*, and advanced glycation end products (AGEs) are produced and accumulated in tissues. Accumulation of AGEs *in vivo* has been reported to cause physical, physiological, and visual damage [1)]. Glycation causes cardiovascular diseases [2)], osteoporosis [3)], and dementia [4)], and is considered to be one of the risk factors for aging. Even in the skin, it has been reported that AGEs accumulate in proteins such as keratin in the epidermis [5)] and collagen [6)] and elastin [7)] in the dermis. It is considered that a large accumulation of AGEs in the stratum corneum of the skin causes it to

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Glycative Stress Research 2021; 8(3): 148-155
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lose its texture [8], and AGEs formed in the dermis cause the yellowing of the skin [9] and also decrease skin firmness and elasticity. Thus, skin glycation is involved in apparent aging, and in recent years, "anti-glycation" has been attracting attention as an important factor in aging care.

Similarly, hair is an important factor for maintaining a youthful appearance. Recently, it has been reported that hair is also glycated over time from the root to the tip and exhibits changes in physical properties, such as a decrease in breaking strength [10]. Decreased breaking strength of hair causes deterioration of hair quality, such as hair breakage and split ends, and accelerates its aging in terms of appearance. However, few studies have examined potential therapeutics for the inhibition of glycation, particularly in hair. In this study, we examined the anti-glycation effect of plant extracts to identify materials that inhibit changes in the physical characteristics of hair caused by glycation.

Method

(1) Reagents

The protein used in the glycation models was wool-derived keratin, which was purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Anti-AGE antibody (clone 6D12) was purchased from Transgenic Co., Ltd. (Fukuoka, Japan). The Alexa546-labeled secondary antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Can Get Signal Immunostain Solution was purchased from Toyobo Co., Ltd. (Osaka, Japan). Other reagents were purchased from Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka) and were of analytical grade.

(2) Sample Preparation

Table 1 shows the 26 plant extracts used as cosmetic ingredients in this study. Aminoguanidine (AG), which is an inhibitory agent for glycation reactions, was used as the positive control in the investigations of the inhibitory effects of AGEs.

(3) Verification of the inhibitory effects of the plant extracts on glycation

The glycation Ker-glucose model was employed to verify the inhibitory effects of the plant extracts on glycation [11]. A glycation solution was prepared with 0.1 mol/L phosphate buffer (PB; pH 7.4), 5.0 mol/L glucose, and 5.0 mg/mL keratin; the sample to be tested was then added to this solution. Subsequently, the glycation solution was reacted at 60 °C for 10 days, and AGE-derived fluorescence was measured using a microplate reader (excitation wavelength 370 nm/detection wavelength 440 nm). A reference (ref) was prepared with the same amount of purified water in place of the samples and reacted under the same conditions. As a positive control for fluorescent AGEs, 1.0 mg/mL of AG was added in the same amount as the sample. The fluorescence intensity was calculated as a relative value when the fluorescence intensity of 5.0 µg/mL quinine sulfate was 1,000. The fluorescence AGE formation inhibitory rate was calculated using the following formula:

The ratio of inhibitory effect on fluorescent AGE formation (%) = 100 – {(sample Glucose (+) – sample Glucose (–)) / (ref Glucose (+) – ref Glucose (–)) × 100},

(4) Primary screening

In the primary screening, each sample was adjusted to a final concentration of 0.1 mg/mL with purified water, and the ratio of the inhibitory effect on AGE formation was measured.

(5) Secondary screening

In the secondary screening, the half maximal inhibitory concentration (IC_{50}) was calculated and evaluated for samples with a fluorescence AGE production inhibitory rate of 75 % or more obtained in the primary screening. Calibration curves for IC_{50} were constructed by adding individual samples to a reaction solution at three concentrations (0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL) and calculating the inhibition of AGE formation after the reaction.

(6) Verification of the inhibitory effects on hair glycation

Hair was collected from healthy women in their 30s who had no history of beauty treatment (bleach, hair color, permanent wave, straightening) with all participants providing an informed consent in writing. Hair was glycated *in vitro* by reacting 1.2 mol/L of glucose and 0.1 mol/L each sample in PB at 50 °C for 10 days. As a control (reference: ref), an equal amount of purified water was added instead of the sample, and the reaction was performed under the same conditions.

(7) Measurement of AGEs in hair samples

Each hair sample was immersed in a degreasing agent [chloroform : methanol (MeOH) = 2:1] at room temperature for 16 hours. The hair was removed from the degreasing agent, dried at room temperature for 1 hour or longer, and then cut to a length of 5 mm or less. Following this, 10 mg of each hair sample was added to 500 µL extraction buffer, which was exposed to 5 M urea, 2.6 M thiourea, 25 mmol/L Tris-HCL buffer, and 5 % 3-mercapto-1, 2-propanediol (pH 8.5), and then reacted at 50 °C for 1 d with stirring. The sample was then centrifuged at 10,000 rpm for 1 minute at 20 °C, and the resulting supernatant was recovered as a protein solution. The fluorescent AGEs of the protein solution were measured using the fluorescence method in the same manner as described above (3). Protein concentration was measured using the Bradford method [12]. Fluorescent AGE content was calculated per 1 mg of the protein content. The difference between the glucose-added group and the non-glucose-added group was evaluated as the amount of change in AGEs levels per protein.

(8) Hair breaking strength measurement

The tensile strength of the hair was measured using a tensile tester (KES-G1-SH single hair tensile tester, Kato Tech Co., Ltd., Kyoto) at a temperature of 25 °C and 65 %

Table 1. Sample profile.

ID	Sample Name	Scientific Name	Family Name	Japanese name	INCI	Extractants
1	Chinese blackberry	*Rubus Suavissimus*	Rosaceae	Ten-cha	Rubus Suavissimus (Raspberry) Leaf Extract	Water
2	Dokudami (BG)	*Houttuynia Cordata*	Saururaceae	Dokudami	Houttuynia Cordata Extract	Butylene Glycol
3	Dokudami (Alcohol)					Alcohol
4	Dokudami (Water)					Water
5	Kuma bamboo grass	*Sasa Veitchii*	Poaceae	Kuma-zasa	Sasa Veitchii Leaf Extract	Alcohol
6	Rooibos	*Aspalathus Linearis*	Fabaceae	Rooibos	Aspalathus Linearis Leaf Extract	Butylene Glycol
7	Evening primrose	*Oenothera biennis*	Onagraceae	Mematuyoigusa	Oenothera Biennis (Evening Primrose) Seed Extract	Water
8	Jiaogulan	*Gynostemma pentaphyllum*	Cucurbitaceae	Amatyaduru	Gynostemma Pentaphyllum Leaf Extract	Water
9	Lemon grass	*Cymbopogon citratus*	Poaceae	Lemon grass	Cymbopogon Schoenanthus Leaf/Stem Extract	Butylene Glycol
10	Mugwort	*Artemisia Princeps*	Asteraceae	Yomogi	Artemisia Princeps Leaf Extract	Alcohol
11	Field Horsetail	*Equisetum Arvense*	Equisetaceae	Sugina	Equisetum Arvense Extract	Alcohol
12	hatomugi	*Coix Lacryma-Jobi*	Poaceae	Hatomugi	Coix Lacryma-Jobi Ma-yuen Seed Extract	Butylene Glycol
13	Loquat (BG)	*Eriobotrya Japonica*	Rosaceae	Biwa	Eriobotrya Japonica Leaf Extract	Butylene Glycol
14	Loquat (Alcohol)					Alcohol
15	Japanese hawthorn	*Crataegus cuneata*	Rosaceae	Sanzasi	Crataegus Oxyacantha Fruit Extract	Alcohol
16	Rose hip	*Rosa canina*	Rosaceae	Rose Hip	Rosa Canina Fruit Extract	Alcohol
17	Chamomile	*Matricaria recutita*	Asteraceae	Kamiture	Chamomilla Recutita (Matricaria) Flower Extract	Alcohol
18	Aloe	*Aloe Arborescens*	Asphodelaceae	Aloe	Aloe Arborescens Leaf Extract	Butylene Glycol
19	Safflower	*Carthamus Tinctorius*	Asteraceae	Benibana	Carthamus Tinctorius (Safflower) Flower Extract	Alcohol
20	Ashitaba	*Angelica keiskei*	Apiaceae	Ashitaba	Angelica Keiskei Leaf/Stem Extract	Butylene Glycol
21	Ginger	*Zingiber Officinale*	Zingiberaceae	Shouga	Zingiber Officinale（Ginger） Rhizome Extract	Alcohol
22	Edible burdock	*Arctium lappa*	Asteraceae	Gobou	Arctium Lappa Root Extract	Alcohol
23	Asian Ginseng	*Panax Ginseng*	Araliaceae	Otane ninjin	Panax Ginseng Root Extract	Alcohol
24	Green tea	*Camellia Sinensis*	Theaceae	Ryoku-cha	Camellia Sinensis Leaf Extract	Alcohol
25	Black tea			Kou-cha		Alcohol
26	Oolong tea			Oolong-cha		Alcohol
Ref	Aminoguanidine	–	–	–	–	–

relative humidity. The cross-sectional area of the hair was measured using a hair diameter measuring device (SK2000 Kato Tech Co., Ltd.), and the breaking strength of the hair was calculated using the following formula:

Breaking strength = Breaking load / Hair cross-sectional area.

(9) Immunofluorescence staining

Immunofluorescence staining was performed using the ABC method with the monoclonal antibody clone 6D12 [8]. The glycated hair was cut to a thickness of 5 μm using a microtome (REM-710 Yamato Scientific Co., Ltd., Asaka, Saitama, Japan), and the sections were fixed on a glass slide. The primary antibody was diluted 1/200 in Can Get Signal Immunostain Solution A and reacted with the hair overnight at 4 °C. Then, the secondary antibody was diluted in the same manner as the primary antibody and reacted with the hair at room temperature for 1 hour. After washing with PB, fluorescence images were obtained using a fluorescence microscope (BZ-X810, Keyence, Osaka). The exposure time was set as the time during which autofluorescence was not detected in the unglycated hair.

Result

(1) Primary screening results

Fig. 1 shows the inhibitory rate of fluorescent AGE formation in each sample. All 26 samples inhibited the formation of fluorescent AGEs. In addition, 75% or more of the inhibitory effect of fluorescent AGE production was observed in 9 of the 26 samples.

(2) Secondary screening results

The IC_{50} values are shown in ***Fig. 2***; Rooibos extract had the highest anti-glycation activity, followed by the green tea and chamomile extracts. In particular, rooibos extract showed a high anti-glycation effect, which was twice as high as that of the green tea extract and more than 20 times higher than that of AG. Among the IC_{50} results, the upper rooibos extract, middle tencha extract (Chinese blackberry), and lower kocha extract (black tea) were used to verify the anti-glycation effect on hair.

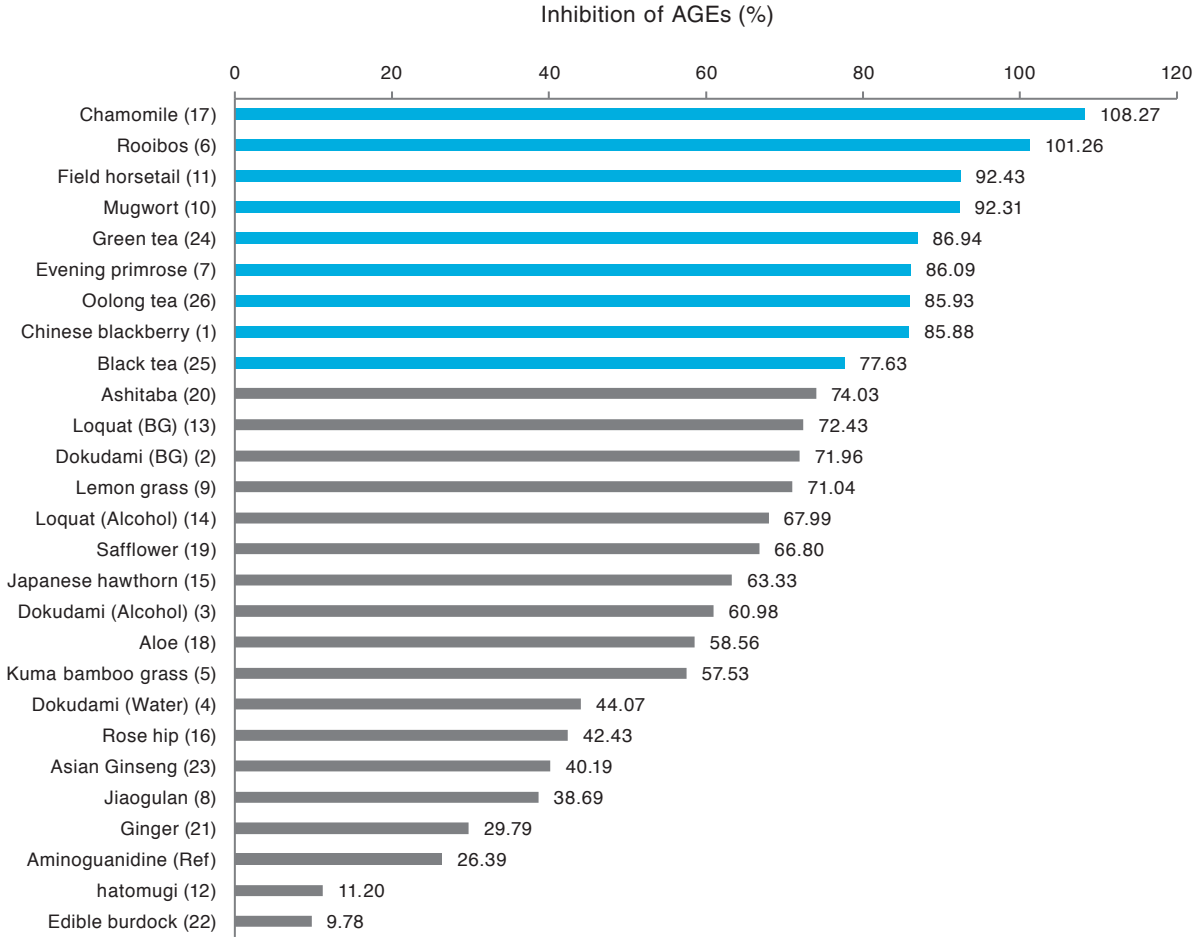

Fig. 1. **Inhibitory effect of plant extracts on AGEs in the Ker-Glucose reaction model.**

The results are expressed as means. 0.1 mg/mL plant extracts were introduced into glycation models containing 5.0 mg/mL Ker and 1.2 mol/L glucose (n = 3). After 10 days of incubation at 60°C, fluorescent AGEs were measured by excitation at 370 nm and emission at 440 nm. AGEs, advanced glycation end products; Ker, keratin.

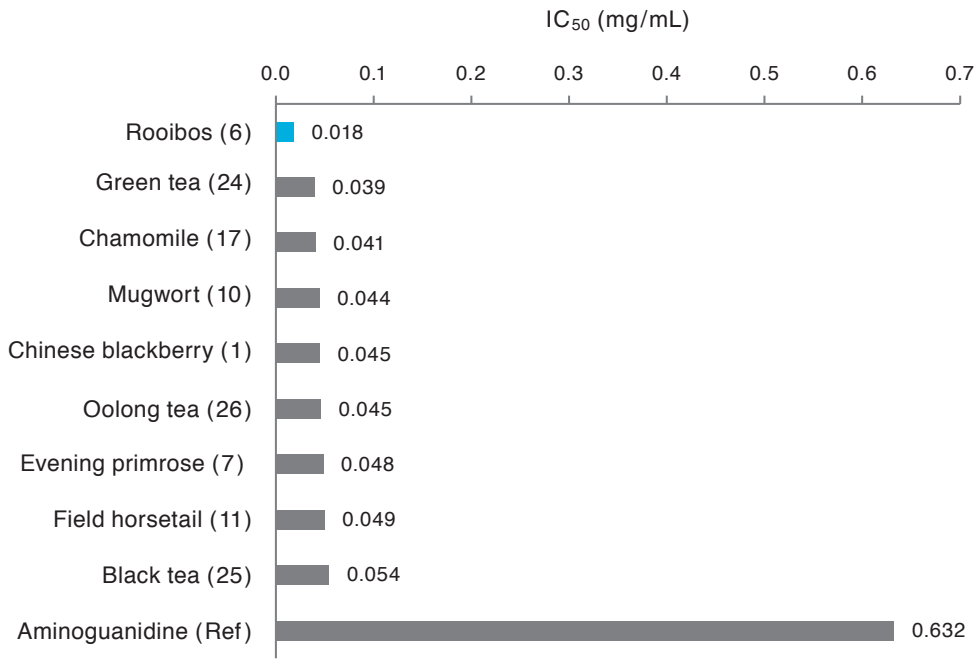

Fig. 2. **IC_{50} of plant extracts on AGEs in the Ker-Glucose reaction model.**

The results are expressed as means. 0.1 mg/mL, 0.01 mg/mL, and 0.001 mg/mL plant extracts were introduced into glycation models containing 5.0 mg/mL Ker and 1.2 mol/L glucose (n = 3). After 10 days of incubation at 60°C, fluorescent AGEs were measured by excitation at 370 nm and emission at 440 nm. IC_{50}, half maximal inhibitory concentration; AGEs, advanced glycation end products; Ker, keratin.

(3) Anti-glycation effects on hair.

Fig. 3 shows the verification results for the anti-glycation effect of the screened samples on hair. The amount of AGEs accumulated in the hair increased with the number of days of *in vitro* glycation. Rooibos extract strongly inhibited the production of fluorescent AGEs (inhibitory rate, 82.4%). In addition, tencha extract and kocha extract also showed a higher production inhibitory effect than AG, and the strength of the effect showed the same trend as the IC_{50} value.

(4) Breaking strength measurements

Fig. 4 shows the results the breaking strength of hair. The breaking strength of the glycated hair was 20.8% lower than that of the unglycated hair. On the other hand, the addition of rooibos extract inhibited the decrease in breaking strength due to glycation to 4.9%.

(5) Fluorescence analysis results

Fig. 5 shows an image of immunofluorescent staining of hair. The accumulation of AGEs in hair due to *in vitro* glycation was observed by fluorescence. It was observed not only in the cuticle on the surface but also in the medula and cortex. The addition of rooibos extract inhibited the formation of AGEs in the entire hair.

Discussion

Screening method using a keratin-glucose glycation reaction model

To date, anti-glycation effects on proteins such as bovine serum albumin (BSA) and human serum albumins (HSA) have been reported for more than 500 types of materials[13-17]. However, it has been reported that the amount of AGEs produced differs depending on the type of protein, and the anti-glycation effect differs depending on the target protein[16]. Therefore, in this study, we first screened for keratin, a protein that constitutes hair. After that, the anti-glycation effects of the plant extracts on keratin were confirmed by screening and verified further using actual hair. Rooibos extract strongly inhibited the production of fluorescent AGEs in both keratin and hair. This result indicates that the keratin-glucose glycation reaction model may be useful for screening materials for hair anti-glycation activities.

Relationship between glycation and hair breaking strength

Glycation is caused not only by reducing sugars, but also by various aldehydes derived from lipids and alcohols[18]. These aldehydes react with proteins to produce AGEs and carbonized proteins, causing various disorders in the body. Recently, the effects of these aldehydes on hair have been reported. One is that glyoxal and glyoxylic acid cause

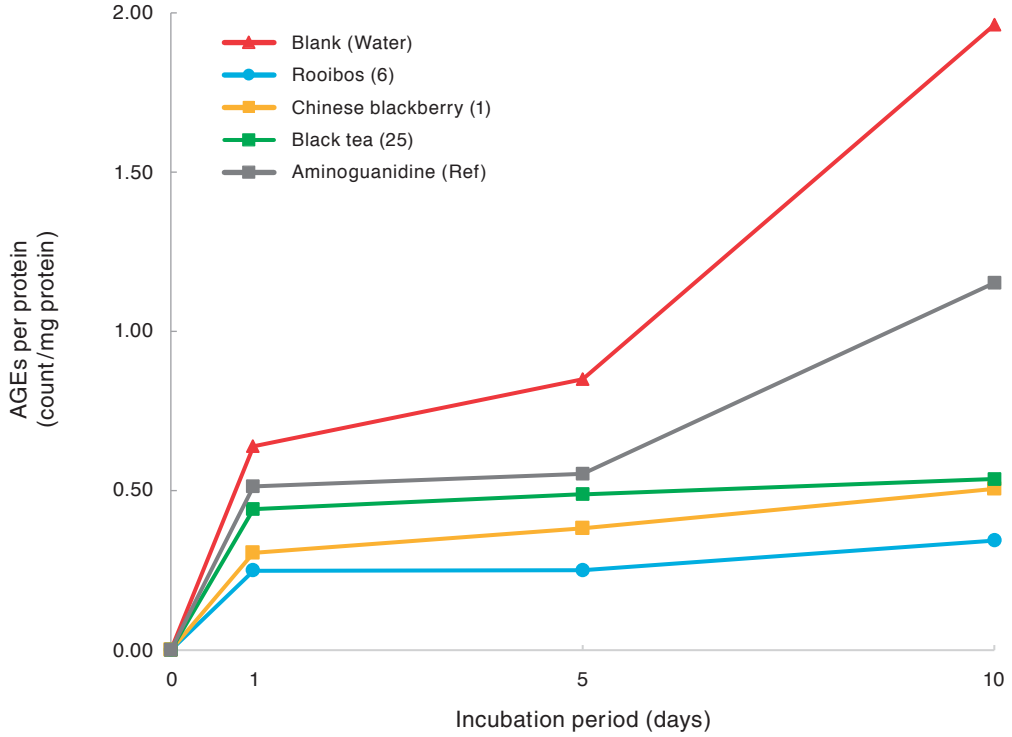

Fig. 3. **AGEs in hair protein.**

The results are expressed as means. Hair samples were introduced into PB containing 1.2 mol/L glucose and 0.1 mg/mL samples. After 10 days of incubation at 50 °C, fluorescent AGEs were measured by excitation at 370 nm and emission at 440 nm. The degree of AGE content was calculated per 1 mg of protein content. AGEs, advanced glycation end products; PB, phosphate buffer.

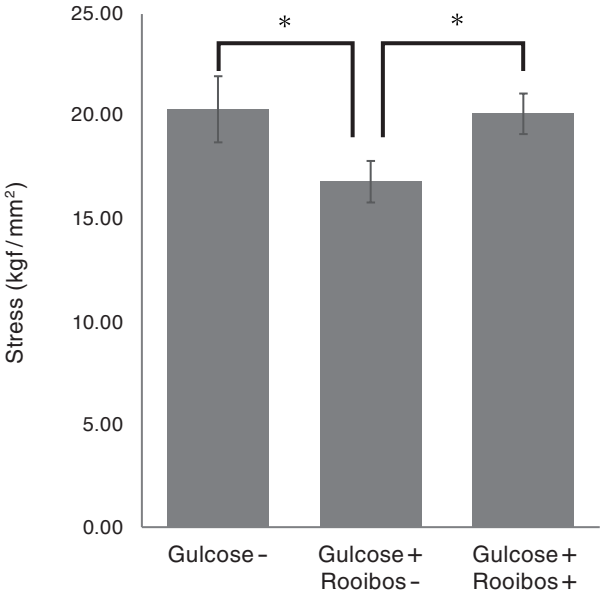

Fig. 4. **Tensile strength of hair.**

Results are expressed as mean ± SD. * $p < 0.05$, Tukey (Kramer test). Hair samples were introduced into PB containing 1.2 mol/L glucose and 0.1 mg/mL samples. After 10 days of incubation at 50 °C, the tensile strength was measured using a high-sensitivity hair rheology analyzer at breaking strength, when the hair was torn off. PB, phosphate buffer; SD, standard deviation.

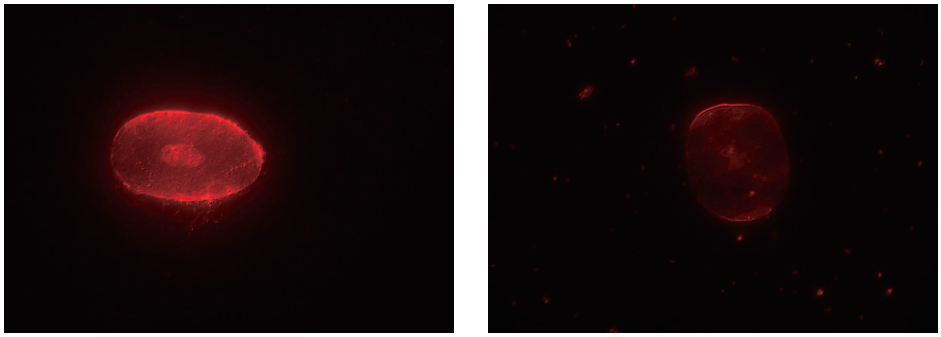

Fig. 5. **Immunofluorescent staining of hair.**

Hair samples were introduced into PB containing 1.2 mol/L glucose and 0.1 mg/mL samples. After 10 days of incubation at 50°C, AGEs were immunostained with an anti-AGE antibody. AGEs, advanced glycation end products; PB, phosphate buffer.

disturbances in the keratin structure of hair and reduce the tensile strength of hair [19]. In addition, glyoxylic acid-treated yak hair rearranges the secondary structure distribution, changes the conformation of disulfide bridges, reduces serine residues, and forms imines [20]. Glyoxal and glyoxylic acid contain aldehyde groups as well as glucose. Therefore, it can be concluded that the above reaction is similar to glycation. This way, the effect of glycation on hair extends to complex and detailed structures, and as a result, changes in physical properties (such as a decrease in breaking strength) occur. In the future, it will be necessary to consider the detailed mechanisms.

Anti-glycation effect of rooibos extract

The rooibos plant (*Aspalathus Linearis*) is endemic to a small part of the western coast of the Western Cape province of South Africa. Rooibos tea, which is produced by the fermentation of harvested leaves, is widely used as a health drink in Africa, Europe, and Japan. Rooibos tea has been reported to have biological effects, such as providing relief from digestive diseases such as constipation, stress relief, and allergic symptoms [21]. In terms of nutrition, it is caffeine-free and contains a large amount of minerals such as potassium, sodium, and magnesium.

Furthermore, it contains various polyphenols, such as rutin, luteolin, quercetin, aspalathin [22]. Luteolin is a flavone and has been reported to have an anti-glycation effect on BSA [23]. Quercetin, a flavonol, has been reported to enhance the inhibitory effect of sulforaphane on the formation of AGEs [24]. These reports suggest that polyphenol components in rooibos inhibit glycation in hair. Among the various components contained in rooibos extract, the component with the anti-glycation effect on hair requires further verification in the future.

Conclusion

In this study, we found that rooibos extract is a novel ingredient that prevents hair glycation. The use of hair cosmetics containing rooibos extract can prevent glycation and maintain beautiful and strong hair.

Conflict of Interest Declaration

Research support was provided by Arimino Co., Ltd.

Acknowledgments

A part of this paper was presented at the 18th Meeting of the Society for Glycative Stress Research (August 31, 2019, Kyoto) and IFSCC Mexico Conference 2021 (October 18-28, 2021, web lecture). The publication of this study was supported by the Research Foundation of Crude Drug gained from the same source of Medicine & Food. (IDF#21007; Chuo-ku, Tokyo).

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