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Original article

Antiglycative effect of black galangal, *Kaempferia parviflora* Wall. Ex. Baker (Zingiberaceae)

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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 in the body is called anti-glycation, and includes suppression of postprandial hyperglycemia, suppression of glycative reaction, and decomposition and excretion of AGEs. It is already known that plant materials, *i.e.*, vegetables and herbs, have an anti-glycative effect. Of these, the rhizome of black galangal (*Kaempferia parviflora* Wall. Ex. Baker; KP), which is a type of ginger family plant (Zingiberaceae), has an action of suppressing the formation of fluorescent AGEs, and polymethoxy flavonoids (PMF); and hydrophilic components are thought to be involved as the active components. In this study, for the purpose of further verifying the possibility of KP as an anti-glycation material, we focused on the hydrophilic component in the KP rhizome and evaluated the anti-glycative effect and AGE degradation action. As a sample, a hot water (80 °C) extract of dry KP rhizome was used. The anti-glycative effect of the KP extract was examined for actions of inhibiting the formation of AGEs and intermediates in the human serum albumin (HSA)-glucose glycation model. Regarding the degrading action of AGEs, the cleaving action of AGE cross-links and the enhancing action of oxidized protein hydrolase (OPH) activity. In the results, the KP extracts inhibited the formation of fluorescent AGEs, pentosidine, CML (N^{ε}-carboxymethyllysine) and intermediates, 3-deoxyglucosone (3DG), glyoxal (GO) and methylglyoxal (MGO). For the degradation effect, the KP extract enhanced actions of both cross-link cleaving of AGEs and OPH activity. In conclusion, it is indicated that KP, as an anti-glycative material, can suppress the production of many types of AGEs with different pathways and acts on the degradation of AGEs, thus reducing the amount of accumulated AGEs in the body.

***KEY WORDS*:** advanced glycation endproducts (AGEs), anti-glycation, pentosidine, N^{ε}-carboxymethyllysine (CML), AGE-crosslink cleavage, oxidized protein hydrolase (OPH)

Introduction

Non-enzymatic binding of protein to glucose produces advanced glycation end products (AGEs). The accumulation of AGEs *in vivo* is a factor in the onset of aging and lifestyle-related diseases. These are called glycative stress, which is a comprehensive combination of the stress reaction on the living body caused by reducing sugar and aldehyde loading and subsequent reactions. On the other hand, suppression of glycative stress in the living body is called anti-glycation. Anti-glycation includes suppression of postprandial hyperglycemia, suppression of glycative reaction, and degradation and excretion of AGEs [1, 2)]. Aminoguanidine (AG) has already been reported to have an AGEs production inhibitory effect, thus showing the effects of preventing and inhibiting the progress on nephropathy, retinopathy, and

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neuropathy [3,4]. However, AG has side effects, *i.e.*, anemia, liver damage, and vitamin B6 deficiency, and has not been put into practical use. It has already been reported that food materials such as tea/herbal tea [5], vegetables/herbs [6], fruits [7], and yogurt [8] have inhibitory effects on glycative reaction. In addition, *N*-phenacylthiazolium bromide (PTB) has been reported as a substance that may act on the degradation of AGEs [9]. PTB cleaves the α-diketone structure, which is a type of cross-linked structure of AGEs-modified proteins. Similar effects have been observed with rosmarinic acid [10], ellagitannin [11], and flavonoids [12]. It has also been reported that oxidized protein hydrolase (OPH), which is a type of *in vivo* enzyme, degrades AGEs-degrading proteins [13]. Furthermore, many kinds of herbal extracts have an OPH activity enhancing effect [14].

It has already been reported that ginger (*Zingiber officinale* Roscoe) has an inhibitory effect on glycative reaction [15,16]. Furthermore, the rhizome of black galangal (*Kaempferia parviflora* Wall. Ex. Baker; KP), which is a kind of ginger family plant (Zingiberaceae), has an anti-glycative effect via inhibiting postprandial hyperglycemia [17] and a fluorescent AGEs production [18,19]. The inhibitory effect on the production of fluorescent AGEs in KP rhizomes is reported to involve polymethoxyflavonoids (PMF), which are characteristic components of this plant species, and hydrophilic components [20].

In this study, for the purpose of verifying the potential of KP as an anti-glycation material, we focused on the hydrophilic components in the KP rhizome and evaluated the anti-glycative effect and AGEs degradation action of a hot water extract of KP rhizome powder.

Materials and methods

1) Reagent

The reagents used in the experiment were purchased from the following manufacturers; human serum albumins (HSA, lyophilized powder, ≥ 96%, agarose gel electrophoresis), 40% methylglyoxal solution (MGO), *N*-phenacylthiazolium bromide; PPD) from Sigma-Aldrich Japan (Meguro-ku, Tokyo, Japan); aminoguanidine hydrochloride (AG), epigallocatechin gallate (EGCg), 2,3-diaminonaphthalene (DAN), 40% glyoxal solution (GO), 1-phenyl-1, 2-propanedione (PPD) and rubusoside (Ru) from Fuji Film Wako Pure Chemical Industries, Ltd. (Osaka, Japan); 3-deoxyglucosone (3DG) from Dojin Chemical Research Institute (Kamimashiki-gun, Kumamoto, Japan); acylamino-acid releasing enzyme (AARE) from Takara Bio (Kusatsu, Shiga, Japan); N-acetyl-L-alanine p-nitroanilide (AAPA) from Bachem (Bubendorf, Switzerland); CircuLex CML/N^{ε}-(Carboxymethyl) Lysine ELISA Kit 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) Rhizome sample and extraction conditions of Kaempferia parviflora (KP)

As a sample, dry powder of KP rhizome sold as a supplement product in Japan was used. The KP powder was provided by Rene Co., Ltd (Osaka, Japan). The dry powder sample was extracted by mixing 40 mL of distilled water and 2 g of powder. The condition of hot water extraction was that the mixture was incubated for 60 minutes in a water bath set at 80 °C. The obtained extract was centrifuged at 2,500 rpm (800 × g) for 10 minutes, and then further filtered to obtain a sample solution. The solid content concentration of the sample solution was calculated by placing 5 mL in an aluminum tray, drying and evaporating it at 120 °C for one hour, and then weighing the evaporation residue.

3) HSA-glucose glycation model

The HSA-glucose glycation model was used to verify the inhibitory effect on glycative reaction [21]. Solution (A), a solution mixed sample solution, 0.1 mol/L phosphate buffer (pH 7.4), 40 mg/mL HSA, 2.0 mol/L glucose aqueous solution, and distilled water at a ratio of 1: 5: 2: 1: 1; Solution (B), a solution in which purified water is added instead of the glucose solution of (A); Solution (C), a solution prepared and incubated with purified water instead of the sample solution of (A); Solution (D), purified water instead of the glucose solution of (A) was prepared, followed by incubation at 60 °C for 40 hours. The amounts of AGEs and intermediates, *i.e.*, 3DG, GO, MGO, in the reaction solution after incubation were measured. AG and EGCg were used for the positive control of inhibitory actions on glycative reaction.

4) Measurement of AGEs

For the evaluation of fluorescent AGEs, 200 μL of the glycative 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 previous report [21]. 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 previous report [22]. CML was measured using 30 μL of reaction solution using CircuLex CML/N^{ε}-(Carboxymethyl) lysine ELISA Kit.

5) Measurement of glycative reaction intermediates

The intermediates, 3DG, GO, and MGO, were measured by HPLC after deproteinizing 200 μL of the reaction solution with perchloric acid, adding DAN under alkaline conditions, and labeling according to the previous report [23,24].

6) Calculation of anti-glycative effect

As for the anti-glycative effect, the production inhibition rate (%) of AGEs and intermediates were calculated by the following formula according to the previous report [21].

$$\text{Formation inhibitory rate}(\%) = \{1 - (A - B) / (C - D)\} \times 100$$

Then, a 50% inhibitory concentration (IC_{50}; mg/mL) was calculated from the formation inhibitory rate of the sample with three different concentrations [21,25]. The smaller the IC_{50} value, the stronger the glycative reaction inhibitory effect.

7) AGE crosslink cleavage action

According to previous report [9,12], the AGEs cross-linking and cleavage action 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 for 8 hours, then stopping the reaction by adding 0.7 N hydrochloric acid, followed by measuring the amount of benzoic acid cleaved from PPD by HPLC.

$$\text{AGE Cleavage rate (\%)} = \{ (A - B) / C \} \times 100$$

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.

8) OPH activity enhancing effect

According to the previous report [14], the OPH activity enhancing action was made via mixing AARE as OPH, a sample solution, 0.025 U/mL OPH, 0.025 mol/L AAPA, and 0.12 mol/L Tris-hydrochloric acid buffer (pH 7.4) at a ratio of 1: 1: 2: 21, then incubated at 37°C for one hour, followed by measuring the amount of *p*-nitroaniline (*p*NA) released from AAPA using absorbance meter at 405 nm; (S). As a reference for measuring OPH activity, distilled water was added, instead of the sample solution added to the mixed solution; (R). For the OPH activity enhancing action, Ru was used for the positive control and EGCg was used for the negative control. The OPH activation rate (%) was calculated by the following formula, assuming that the amount of *p*NA generated in 60 minutes immediately after the start of the reaction (0 minutes) in the reference reaction was 100%.

$$\text{OPH activity enhancing rate (\%)} = \{ (S_{60} - S_0) / (R_{60} - R_0) \} \times 100$$

S; *p*NA concentration of sample solution addition reaction solution, R; *p*NA concentration of reference reaction solution, 60; 60 minutes later, 0; immediately after reaction (0 minutes).

Statistical analysis

The measured values are shown as mean ± standard deviation. Tukey's test was used to compare the measured values. As a result of the statistical analysis, a significance level of less than 5% was considered significant.

Result

AGE formation inhibitory effect

The formation inhibitory effects on fluorescent AGEs and CML by the KP extract were found to be concentration-dependent (***Fig.1***). The formation inhibitory rate by KP at a solid content concentration of 0.79 mg/mL was 79.9% for fluorescent AGEs, 52.0% for pentosidine, and 98.8% for

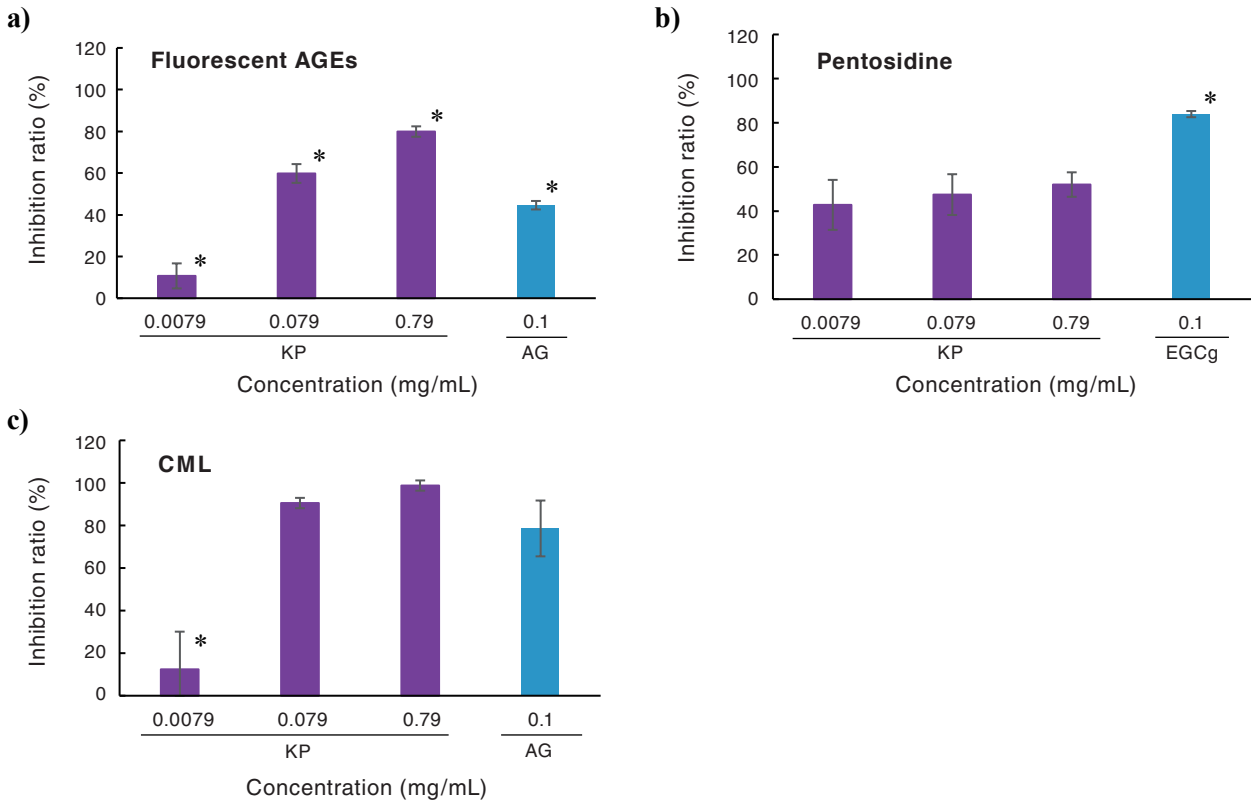

Fig.1. **Inhibitory activity of KP extract on AGE formation.**

a) Fluorescent AGEs, **b)** Pentosidine, **c)** CML. Results are expressed as mean ± standard deviation, n = 3, * $p < 0.05$ by Tukey' s test. KP, *Kaempferia parviflora* rhizome extract; AG, aminoguanidine; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine.

CML. The IC_{50} values were 0.078 mg/mL for fluorescent AGEs, 0.292 mg/mL for pentosidine, and 0.031 mg/mL for CML (***Table 1***). The IC_{50} value of the KP extract was 0.65 times higher for fluorescent AGEs and 0.28 times higher for CML than AG. The formation inhibitory rate of pentosidine by KP was 42.7% to 52.0% at an extract concentration of 0.0079 to 0.79 mg/mL, in which concentration-dependence was not observed. The IC_{50} value of pentosidine was 21.3 times higher in KP extract than in EGCg.

Formation inhibitory actions on glycative reaction intermediates

The formation inhibitory effects on 3DG and GO by the KP extract were found to be concentration-dependent (***Fig. 2***). The formation inhibitory rate by KP with a solid content concentration of 0.79 mg/mL was 89.4% for 3DG and 88.2% for GO. The IC_{50} values were 0.028 mg/mL for 3DG and 0.038 mg/mL for GO (***Table 1***). The IC_{50} value of

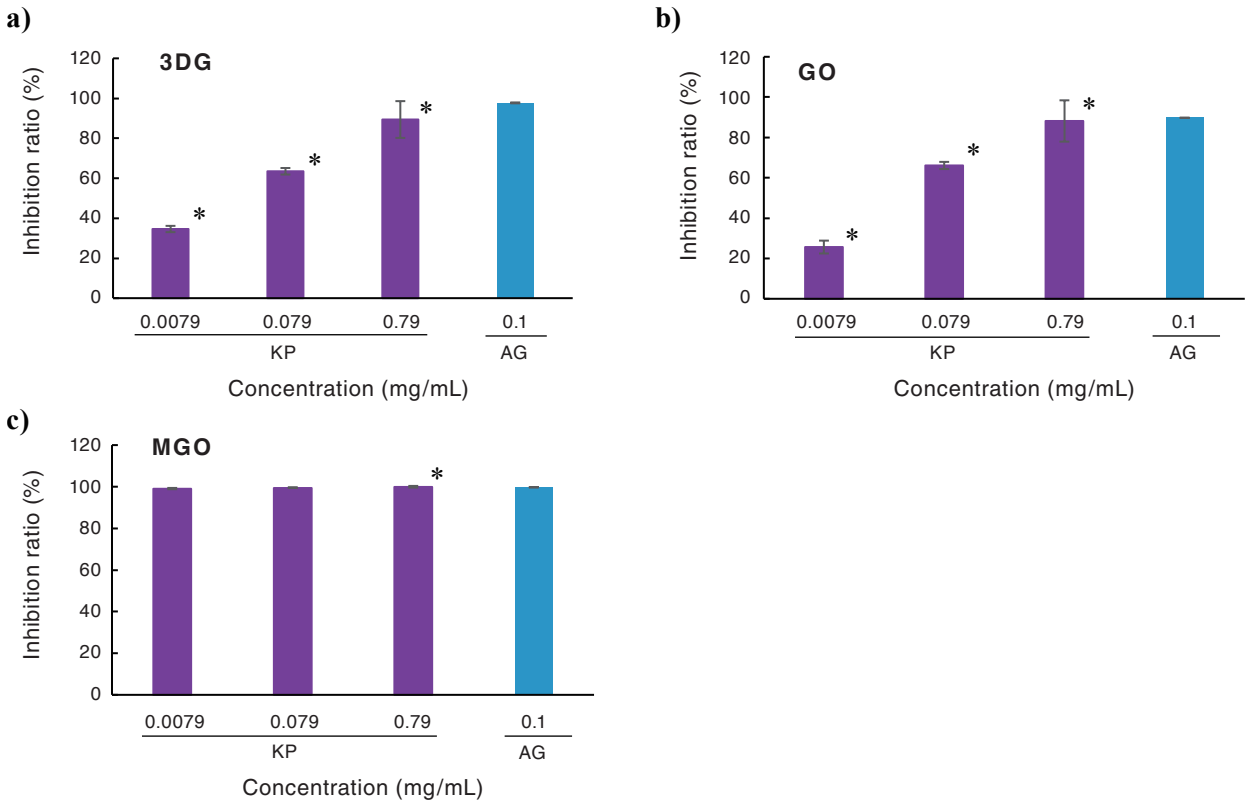

Fig. 2. **Inhibitory activity of KP extract on intermediate of AGE formation.**

a) 3DG, **b)** GO, **c)** MGO. Results are expressed as mean ± standard deviation, n = 3, *; $p < 0.05$ by Tukey' s test. KP, *Kaempferia parviflora* rhizome extract; AG, aminoguanidine; EGCg, epigallocatechin gallate; AGEs, advanced glycation end products; 3DG, 3-deoxyglucosone; GO, glyoxal; MGO, methylglyoxal.

Table 1. Inhibitory activity of KP extract on AGEs and intermediate formation.

Index for anti-glycative effect	KP IC_{50} (mg/mL)	Aminoguanidine IC_{50} (mg/mL)	EGCg IC_{50} (mg/mL)
Fluorencet AGEs	0.078	0.120	–
Pentosidine	0.292	–	0.014
CML	0.031	0.114	–
3DG	0.028	0.126	–
GO	0.038	0.117	–
MGO	<0.010	<0.010	–

KP, *Kaempferia parviflora* rhizome extract; EGCg, epigallocatechin gallate; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; 3DG, 3-deoxyglucosone; GO, glyoxal; MGO, methylglyoxal.

the KP extract was 0.22 times that of 3DG and 0.38 times that of GO compared to AG. The MGO formation inhibitory rate by KP was strong, 99% or more in each of the extract concentrations of 0.0079 to 0.79 mg/mL.

AGE crosslink cleavage action

The AGE crosslink cleavage action was found to be concentration-dependent at KP extract concentrations of 0.13 to 3.94 mg/mL (***Fig. 3***). The cleavage rate at the KP extract concentration of 3.94 mg/mL was 6.81%, which was 0.27 times that of the 5 mmol/mL PTB (cleavage rate 25.5%), a positive control.

OPH activity enhancing effect

The OPH activity enhancing effect was 203.3% at a KP extract concentration of 0.315 mg/mL (***Table 2***). This effect was 0.97 times that of 0.04 mg/mL Ru (acting rate 210.2%), a positive control, and 11.5 times that of 0.04 mg/mL EGCg (acting rate 17.7%), a negative control.

Discussion

Anti-glycative effect by KP

The formation inhibitory rate of fluorescent AGEs, pentosidine, CML, 3DG, GO, and MGO were measured for the purpose of verifying the anti-glycative effect of a hot water extract of KP rhizome powder. As a result, the anti-glycative effect of the KP extract was observed in all the measurement items. IC_{50} values for the fluorescent AGEs, CML, 3DG showed equal to, or higher effect of the KP extract than those of AG, a positive control. AG suppresses the formation of AGEs by blocking carbonyl groups such as intermediates [3]. In a clinical trial in which patients with diabetic nephropathy were administered with AG at 150 mg or 300 mg per day for 42 consecutive months, their urinary protein excretion was reduced by inhibiting AGE formation for 21 months. However, after that, the usefulness was not recognized in the 300 mg intake group [4]. The reason may be that the glycative reaction *in vivo* has multiple pathways, and there are many types of AGEs and intermediates, which are produced during the reaction process [26]. Therefore, in order to inhibit the glycative reaction *in vivo*, it is necessary to suppress the formation of various types of AGEs and intermediates. It has already been verified *in vitro* and in human clinical trials that the mixed herb extract of *Houttuynia cordata*, chamomile, hawthorn, and grape leaves inhibits the glycative reaction in multiple pathways with various components [27-30]. The anti-glycative effect of the KP extract may have the same effect as the mixed herb extract because it suppressed the formation of many types of AGEs with different pathways.

The hydrous ethanol extract of KP has an inhibitory effect on the formation of fluorescent AGEs, and PMF has been reported as an active ingredient [18]. For the purpose of analyzing the functional components responsible for the anti-

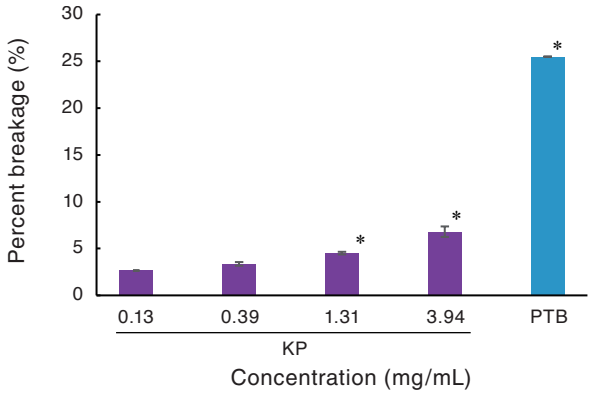

Fig. 3. **AGE cross-link breaking activity.**

Results are expressed as mean ± standard deviation, n = 3, * $p < 0.05$ by Tukey' s test. KP, *Kaempferia parviflora* rhizome extract; PTB, 5 mmol/L *N*-phenacylthiazolium bromide.

Table 2. OPH enhance activity.

Sample	Concentration (mg/mL)	Percent enhancement (%)
KP	0.315	203.3 ± 19.4 *
Rubusoside (positive control)	0.040	210.2 ± 27.2 *
EGCg (negative control)	0.040	17.7 ± 3.2 *
Ref (water)	–	100

Results are expressed as mean ± standard deviation, n = 3, * $p < 0.05$ by Tukey' s test. KP, *Kaempferia parviflora* rhizome extract; EGCg, epigallocatechin gallate; OPH, oxidized protein hydrolase; Ref, reference,

glycative effect, we compared formation inhibitory actions on fluorescent AGEs from ten types of rhizome samples of KP and verified the relationship with PMF contained in KP [20]. As a result, the formation inhibitory effect on fluorescent AGEs was stronger in the hot water extract having a low PMF content than in the 70% ethanol extract containing a large amount of PMF. In this study, the formation inhibitory effects by the KP hot water extract showed not only on fluorescent AGEs but also on pentosidine, CML, 3DG, GO, and MGO. The anti-glycative effect of KP may involve more water-soluble components than hydrophobic components such as PMF.

AGE degradation action by KP

The AGE crosslink cleavage rate and the OPH activity enhancing rate were measured for the purpose of verifying the AGE degradation action by a KP rhizome powder hot water extract. As a result, the KP extract was found to have an AGE crosslink cleavage action and an OPH activity enhancing action.

It has been shown that the PTB used for the positive control in this study degradate PPD to produce benzoic acid, which may cleave AGE-modified protein crosslinks [9,31]. PTB has also been shown to reduce AGEs in rat arterial walls [32]. AGE crosslink cleavage actions have been reported in various plant components [11-13]. KP extracts that act similarly to these may contain similar agents. On the other hand, in a report using the PTB-administered rat model, a reduction in the AGE crosslink of tail collagen has not been observed [33]. Further verification is required for the possibility of AGE decomposition by the components verified by the AGE crosslink cleavage model.

OPH has long been known as an *in vivo* enzyme having the action of degrading and releasing the N-terminal modifying group of modified proteins such as acyl, formyl, and acetylation [34]. Furthermore, OPH has shown to degrade AGE-modified proteins [13]. It is also speculated that OPH and the proteasome act in concert with the degradation of aging proteins [35]. Many types of herbal extracts have been reported as active ingredients that enhance the OPH activity [14]. The OPH activity enhancing effect by herbs differs depending on the site where the material is used and is stronger in roots and seeds than in leaves. The KP used in this study is rhizome and may be associated with the site of action of the herb. OPH is an *in vivo* enzyme present in the liver, brain, and blood. From these facts, KP may be involved in the degradation of aged proteins including glycation in the body.

Conclusion

For the purpose of verifying the potential of KP as an anti-glycative material, the anti-glycative effect and AGE decomposition action of an 80 °C hot water extract of KP rhizome dry powder were verified. The KP extract has been shown to inhibit the formation of fluorescent AGEs, pentosidine, CML and intermediates, 3DG, GO and MGO. In addition, both an AGE crosslink cleavage action and an OPH activity enhancing action were found in the AGE degrading process by the KP extract. It is suggested that KP inhibited the formation of many types of AGEs with different pathways, and decomposed AGEs, thus acting as an anti-glycative material to reduce the amount of AGEs accumulated in the body.

Conflict of interest declaration

In carrying out this research, we received support from Rene Co. Ltd.

Acknowledgment

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