

Original article

Antiglycative effect of *Kaempferia parviflora* Wall. Ex. Baker (Zingiberaceae) : Prevention of advanced glycation end product formation.

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Abstract

Advanced glycation end products (AGEs) are formed due to non-enzymatic reactions between protein and glucose. The in vivo-formed and -accumulated AGEs are a risk factor of accelerating the aging process or causing onsets of lifestyle diseases, which is considered as glycative stress. Some plant materials, such as vegetables and herbs, are recognized to have anti-glycation effects. It has been reported that *Zingiber officinale* Roscoe has an inhibitory effect on glycation. Rhizomes of *Kaempferia parviflora* Wall. Ex. Baker (KP), one of the plants of the *Zingiberaceae* family, have an anti-glycation effect, and polymethoxy flavonoid (PMF) is involved in anti-glycation activities as an active component of KP, as has been reported. This study aimed to analyze active constituents of KP as an inhibitor on glycation, comparing, regarding inhibitory effectiveness, extracts of KP rhizomes sold or marketed as health foods in Japan and examining the correlation between inhibitory effectiveness and 5 types of PMF contained in KP.

Ten types of KP materials and 5 types of PMF were investigated as test samples. Dried powder samples were extracted with hot water at 80°C and with 70% ethanol. Sample extract solution or sample solution was added to the glycation model of human serum albumin (HSA) and glucose. The formed fluorescent AGEs (excitation wavelength 370 nm/detection wavelength 440 nm) were measured, and inhibition rates of AGEs formation were calculated with the measurement values of AGEs. The effectiveness of inhibition was assessed with AGEs at an inhibitory concentration of 50% (IC₅₀). Following this, hot-water extracts of the powder sample were examined with fractionation and purification using hydrophobic cartridge columns and the inhibitory effect on glycation of each effluent fraction were examined. The content of PMF in sample solutions were measured, using HPLC.

Anti-glycation effects were recognized in 4 types of PMF and all of the 10 types of KP materials. Inhibitory effects on glycation tended to be stronger in hot-water extracts of dried powder than in the 70% ethanol extract solution and essence extract solution. Larger amounts of PMF contained in KP were detected in 70% ethanol extracts than in hot-water extracts ($p < 0.03$). There was no recognized correlation between the IC₅₀ of the sample solution and content of the PMF. Analyzing hot-water extracts of dried powder using hydrophobic cartridge columns with fractionation and purification, purified water not containing PMF, 50% acetonitrile containing PMF, and 75% acetonitrile containing PMF showed an anti-glycation effect with eluate fraction.

Hydrophilic substances were assumed to have existed, as anti-glycation constitutes of KP, excluding for PMF. Furthermore, there was a possibility that components, except for PMF, could greatly contribute to the inhibitory effects of KP.

KEY WORDS: anti-glycation effect, *Kaempferia parviflora*, polymethoxy flavonoid

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Introduction

Non-enzymatic glycation, which is the bonding of reducing sugar such as glucose to protein, forms advanced glycation end products (AGEs). The *in vivo* formation and accumulation of advanced glycation end products induces glycative stress, accelerating the aging process or causing an onset risk of lifestyle diseases ^{1, 2}. The inhibition of postprandial hyperglycemia, the inhibition of glycation and the degradation and excretion of AGEs are measures for reducing glycative stress. It has previously been reported that aminoguanidine (AG) has inhibitory effects of AGEs formation and effects of preventing disease progression of nephropathy, retinopathy and neuropathy ^{3, 4}. However, AG has not reached a stage for commercialization, as AG was found to have adverse effects, such as anemia, hepatopathy and vitamin B6 deficiency. It has been reported that tea, herb tea ⁵, vegetables, herbs ⁶, fruits ⁷ and yoghurt ⁸ are foods with inhibitory effects on glycation. Furthermore, *Zingiber officinale* Roscoe, which has long been utilized as a vegetable and herbal medicine, have inhibitory effects on glycation, as has been reported ^{9, 10}. Rhizomes of *Kaempferia parviflora* Wall. Ex. Baker (KP), one of the plants of the *Zingiberaceae* family, has been utilized recently as a health food. KP is characterized by the blackish purple of the root inside, and its products sold in Japan are called black galangal, black ginger or black turmeric. KP is distributed in Thailand, Laos, India and Myanmar. The appellation of origin KP in Thailand is Krachai Dam ¹¹. The extracted liquid of KP roots in Bangladesh is traditionally utilized as an alternative medicine for vomiting and diarrhea ¹². A decoction of KP with alcohol is a traditional medical treatment for asthma, erectile dysfunction, gout, diarrhea, dysentery and diabetes mellitus ^{13, 14}. It has been reported that extracts of KP roots function for the activation of lipid metabolism, brown adipose tissue and lipolysis of white adipose tissue ¹⁵. The characteristic components contained in KP roots are anthocyanin and polymethoxy flavonoid (PMF).

It has been reported that PMFs, which are contained in KP, have effects to improve metabolic syndromes, the metabolic function of muscle ¹⁵ and obesity ¹⁶, as well as to enhance physical fitness ^{17, 18}. KP roots have anti-glycation effects, and PMFs are involved in its action component ¹⁹.

This study aimed to analyze active constituents of KP as an inhibitor on glycation, comparing, regarding inhibitory effectiveness, extracts of KP rhizomes sold as health foods in Japan and examining correlations between inhibitory effectiveness and PMF contained in KP.

Materials and methods

1) Reagent

Human serum albumins (HSA), lyophilized powder, ≥ 96% (agarose gel electrophoresis) was purchased from Sigma-Aldrich Japan (Meguro-ku, Tokyo, Japan). Aminoguanidine hydrochloride (AG) was purchased from Wako Pure Chemical Industries (Chu-ku, Osaka, Japan). Reagents of 5,7 - dimethoxyflavone, 5,7,4' - trimethoxyflavone, 3,5,7 - trimethoxyflavone, 3,5,7,4' - tetramethoxyflavone and 3,5,7,3',4' - pentamethoxyflavone were purchased from TOKIWA Phytochemical (Sakura, Chiba, Japan). Other reagents of first or HPLC grade were purchased from Wako Pure Chemical Industries or nacalai tesque (Nakagyo-ku, Kyoto, Japan).

2) Rhizome of *Kaempferia parviflora* (KP)

Samples of 10 types of KP rhizomes were KP extracts sold in Japan: dried matters and essence extract powder ([Table 1](#)). Powdery samples were used with no alteration, and slice dried samples were used after being crushed to powder by a laboratory mixer (Osaka Chemical, Kita-ku, Osaka, Japan).

Table 1. The rhizome sample of *Kaempferia parviflora*.

Sample number	Sample form	Product name	Maker	Manufacturing method	Solid content
v216-1	dried	black galangal (cooled-vacuum drying)	RENE Co., Ltd., Osaka Japan	dried (35°C, -98 MPa) rhizome powder	–
v216-2		black galangal (hot-air drying)	RENE Co., Ltd., Osaka Japan	dried (100°C, air) rhizome powder	–
v219		black ginger powder - MF5	JAPAN TBLET Co., Ltd., Kyoto Japan	dried rhizome powder	–
v220		black ginger (Krachaidum)	AKAJIN Co., Ltd.	dried slice rhizome	–
v217	extract	black ginger extract - WSP	ORYZA OIL & FAT CHEMICAL Co., Ltd., Aichi Japan	hydrous ethanol extract	5%
v218		black ginger extract - P	ORYZA OIL & FAT CHEMICAL Co., Ltd., Aichi Japan	hydrous ethanol extract	30%
v221		black ginger extract powder	TOYO SHINYAKU Co., Ltd., Fukuoka Japan		100%
v222		SIRTMAX (black turmeric extract)	TOKIWA Phytochemical Co., Ltd., Chiba Japan	hydrous ethanol extract	100%
v223		black ginger extract	MARUZEN Pharmaceuticals Co., Ltd., Hiroshima Japan		100%
v224		black ginger extract	Sabinsa Japan Corporation, Tokyo Japan		100%

3) Preparation for KP extract

Preparation for dried powder samples was as follows: extracts were obtained with 2 g of powder and a mixture of 40 mL of purified water or 70 % ethanol solution. For hot-water extracts, the mixed solution was incubated for 75 minutes in a water bath where the temperature was maintained at 80°C. For 70 % ethanol extracts, the mixed solution was incubated for 4 hours in a water bath at 50°C. Samples were obtained after both extract solutions were centrifuged at 2,500 rpm (800×g) for 10 minutes and filtered. To measure the concentrations of the solid samples, 5 mL of the sample solutions were put in an aluminum tray and then dried and vaporized at 120°C for 1 hour. Concentrations of the solid samples were calculated by weighing the amount of evaporation residues. For the preparation of the extract powder, by obtaining as precise information as possible regarding the solid content ratio of the extract powder from the manufacturing makers of the samples, the samples of extract powder were dissolved at the level of 10 mg/mL of solid concentration, adding a 70 % ethanol solution.

4) Measurement of anti-glycation activity

The measurement of anti-glycation activities employed the glycation model of HSA and glucose²⁰. The inhibitory concentrations of the AGE formation were measured as follows: Solution A was a mixture of the sample solution, 0.1 mol/L phosphoric acid buffer (pH 7.4), 40 mg/mL HSA, 2.0 mol/L glucose solution and distilled water at a ratio of 1 : 5 : 2 : 1 : 1. Solution B was a mixed solution containing purified water instead of glucose solution in Solution A. Solution C was an incubated solution which was arranged with purified water instead of the sample solution in Solution A. Solution D was a mixed solution containing purified water instead of the glucose solution in Solution C, which was incubated at 60°C for 40 hours. To measure the volume of the AGEs of the reaction liquid after incubation, a fluorescent microplate reader was employed, measuring the AGE-derived fluorescence (excitation wavelength: 370 nm; detection wavelength: 440 nm). The inhibitory ratio of the AGEs formation was calculated with the following formula.

$$\text{The inhibitory concentration of AGEs formation (\%)} = [1 - (A - B) / (C - D)] \times 100$$

Furthermore, a 50 % inhibitory concentration (IC₅₀) was used; mg/mL was calculated based on the inhibitory rate of the AGE formation of each KP extract solution at three concentrations²¹. Smaller values of IC₅₀ indicate a stronger anti-glycation effect.

5) Measurement of polymethoxylated flavonoid (PMF)

After being diluted two times, PMF was measured using HPLC as follows: Column: Inerstil ODS-4, 3 µm, 100 × 4.6 mm I.D. (GL Sciences, Shinjuku-ku Tokyo, Japan), Eluent: 0.1 % formic acid/0.1 % formic acid - acetonitrile (ACN) (70/30), Flow rate: 1.0 mL/min, Detection wavelength: UV at 260 nm, Column temperature: 25°C, Sample injection volume: 10 µL.

6) Fractionation of extracts using hydrophobic cartridge column

Fractionation and purification of hot-water extracts employed hydrophobic cartridge column (Oasis HLB, Nihon Waters, Shinagawa-ku, Tokyo, Japan). Hydrophobic cartridge columns were conditioned with 3 mL of ACN and 3 mL of distilled water. Following this step, 1 mL of the sample solution was injected. Subsequently, 3 mL of elution was examined step by step with ACN concentrations from 0 %, 10 %, 20 %, 50 % to 75 %, and then collected.

Statistical analysis

The inhibitory rates of AGE formation were shown as mean value ± standard deviation. Comparisons between groups were performed in measurements using the Tukey's test. The correlation analysis between the measured values was performed using the Pearson's correlation coefficient. Interpretation of correlation was at a range of $0.4 < |r| \leq 1.0$, and weak correlation at a range of $-0.2 < |r| \leq 0.4$. A hazard rate of less than 5 % was considered to be statistically significant.

Results

Anti-glycation effect of KP

Ten types of KP samples were examined: 4 types of dried powder of rhizomes and 6 types of essence extract powder of rhizomes. The IC₅₀ of each sample as anti-glycation activities was shown, classifying each into hot-water extract of dried powder, 70 % ethanol extract solution, and extract powder (**Fig. 1**).

The values of the IC₅₀ were 0.027 ± 0.010 mg/mL (mean ± standard deviation, 4 samples) in the hot-water extracts, 0.029 ± 0.011 mg/mL (4 samples) in the 70 % ethanol extracts, and 0.064 ± 0.053 mg/mL (6 samples) in the powder extracts. The mean value of the hot-water extracts was the smallest. However, there was no statistically significant difference in the measured values of the extracts among the 3 groups.

All of the IC₅₀ in the hot-water extracts and 70 % ethanol extracts of the 4 types of dried powder samples were smaller than in AG. Particularly, the IC₅₀ (0.014 mg/mL) of the v216-1 in the hot-water extraction had the smallest value of the 10 types of KP materials; the value was 1/3.4 of AG. The IC₅₀ values of the 70 % ethanol extracts were smaller than the AG, except for v244.

Anti-glycation activity of PMF

The inhibition rate of the AGE formation was measured in 5 types of PMF contained in KP: 5,7 - dimethoxyflavone, 5,7,4' - trimethoxyflavone, 3,5,7 - trimethoxyflavone, 3,5,7,4' - tetramethoxyflavone and 3,5,7,3',4' - pentamethoxyflavone. As a result, in the 4 types of PMF, excluding that of 3,5,7 - trimethoxyflavone, anti-glycation activity was recognized (IC₅₀; 0.028 ± 0.024 mg/mL, 4 samples, **Table 2**). There was no correlation between the IC₅₀ and the number, or the binding site of the methoxy groups within the PMF molecule.

The amount of PMF in the KP extract solution

The amount of PMF in the hot-water extracts of dried powder sample (0.20 ± 0.04 mg/mL, 4 samples) were 3.6 times (v220) - 8.2 (v216-1) times larger than that in the ethanol extracts (1.11 ± 0.54 mg/mL, 4 samples, $p < 0.03$, Fig. 2). The amount of PMF in the extract powder solution was recorded to be 5.7 times larger than the differences, depending on the types of samples. Composition ratios of the 5 types of PMF contained in the sample extract solution were almost the same both in the ethanol extract solution and extract powder solution. However, regarding the hot-water extract, the ratio of 3,5,7 - trimethoxyflavone was one-sixth of others (1.3 ± 2.6 mg/mL, 4 samples).

Correlation between the volume of PMF and the anti-glycation activity of KP extract solution

There was no correlation between the total amount of PMF contained in KP (except for 3,5,7 - trimethoxyflavone),

and IC_{50} of KP (Fig. 3). Although hot-water extracts contained less amount of PMF content in comparison with other sample solution, they showed smaller IC_{50} .

Anti-glycation activities of KP extracts in fractionated eluate with hydrophobic cartridge column

A sample of v216-1, which had the smallest IC_{50} in hot-water extraction for dried powder, was examined with fractionation and purification of the hydrophobic cartridge column, and then the anti-glycation activity of the eluate was measured. As a result, on water fractions, 50% ACN fractions and 75% ACN fractions, the inhibition ratios of the AGEs formation were more than 50% (Fig. 4). Measurement of the amount of PMF in each eluted fraction indicated that PMF existed in 50% ACN and 75% ACN fractions. The amount of PMF contained in 50% ACN fraction was almost the same as that of 75% ACN. However, the inhibition concentration of the AGEs formation at 75% ACN fraction was 1.8 times stronger than that of 50% ACN ($p < 0.01$).

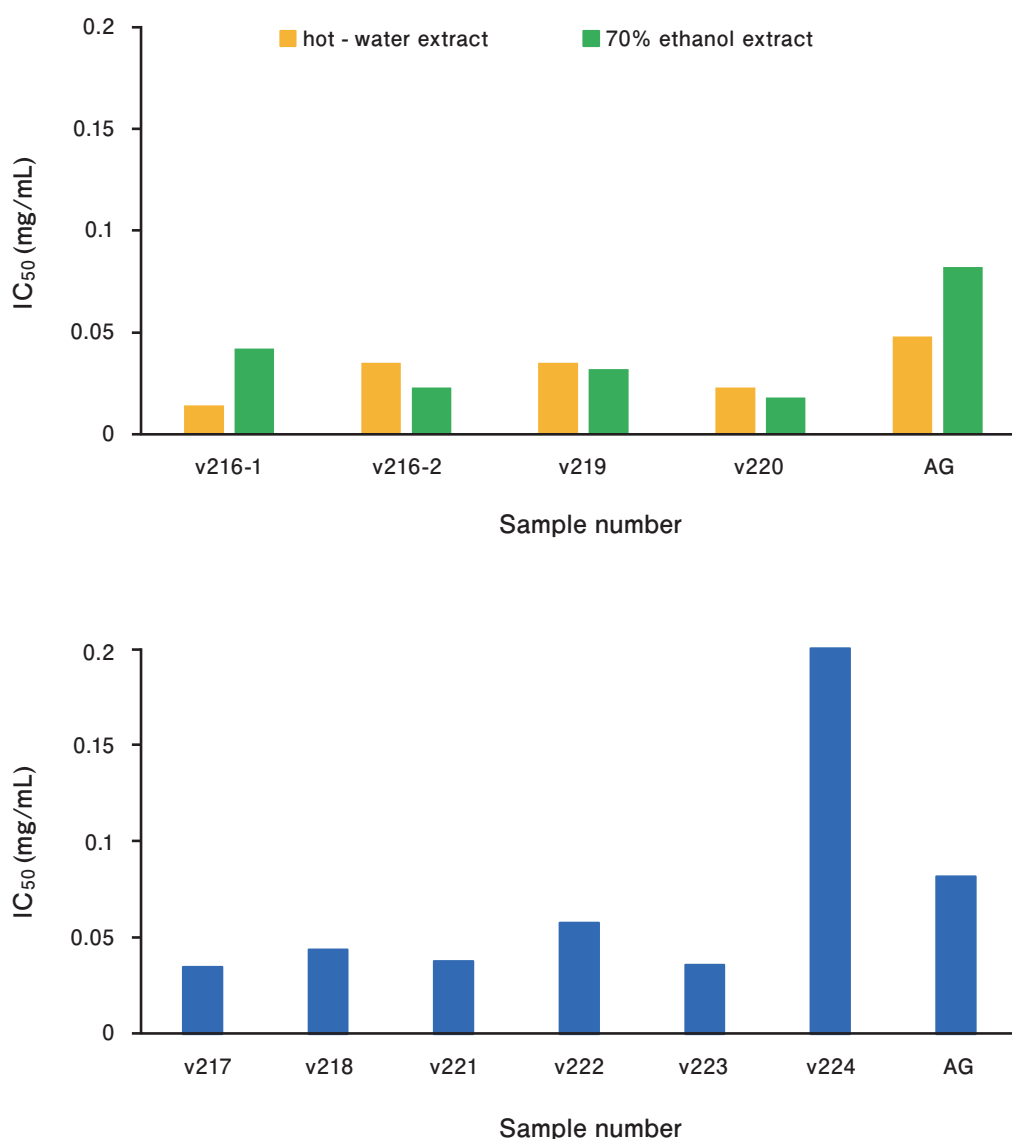


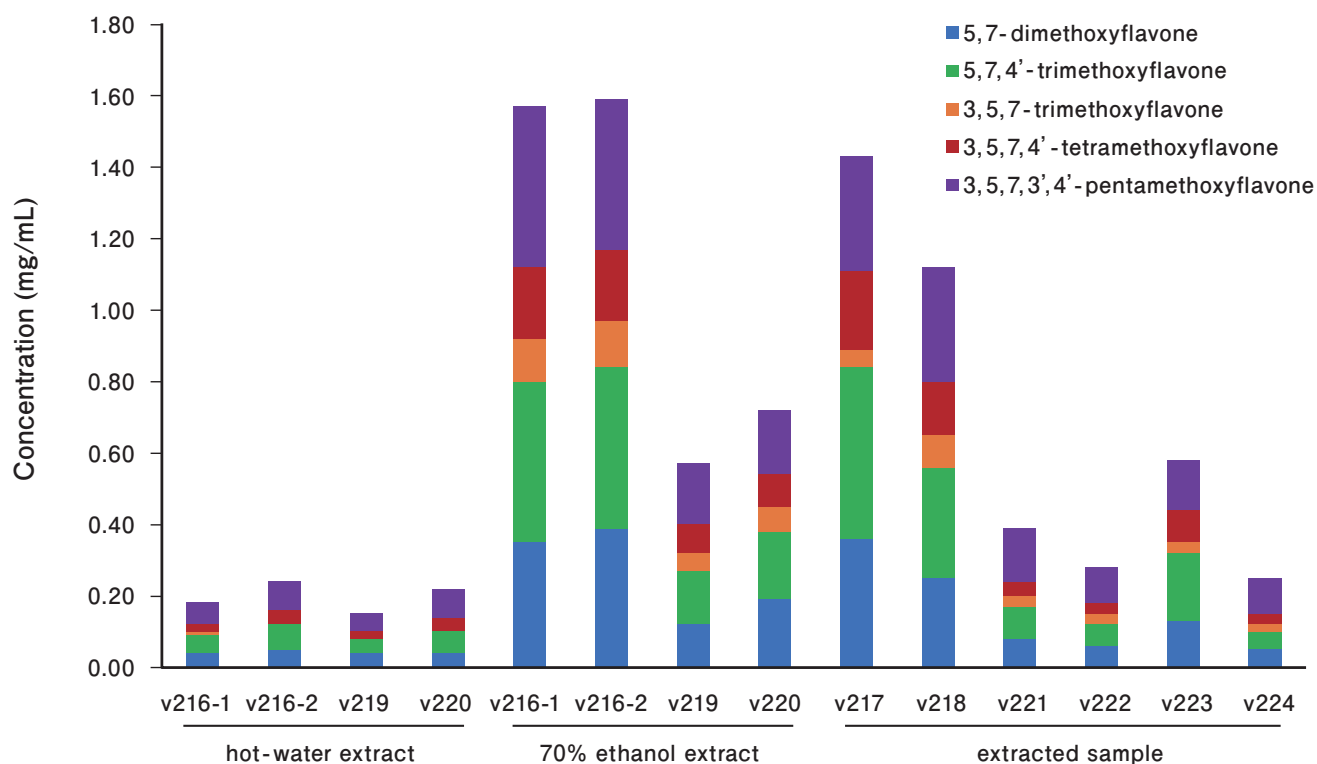
Fig. 1. Anti-glycation activity of *Kaempferia parviflora*.

Sample number; reference of Table 1. IC_{50} calculated HSA-glucose glycation model test result. IC_{50} ; 50% inhibitory concentration of AGE formation; AGE, advanced glycation end product; HSA, human serum albumin.

Table 2. Anti-glycation activity of polymethoxyflavone involvement *Kaempferia parviflora* rhzome.

polymethoxyflavonoid	IC ₅₀ (mg/mL)
5, 7-dimethoxyflavone	0.061
5, 7, 4'-trimethoxyflavone	0.009
3, 5, 7-tridimethoxyflavone	> 0.1
3, 5, 7, 4'-tetramethoxyflavone	0.012
3, 5, 7, 3', 4'-pentamethoxyflavone	0.029

IC₅₀; 50% inhibitory concentration of AGE formation; AGE, advanced glycation end product.

**Fig. 2. Polymethoxyflavonoid concentration of *Kaempferia parviflora* rhzome extract.**

Sample number; reference of **Table 1**. Polymethoxyflavonoid measured by HPLC method. Column; Inerstil ODS-4, 3 μ m, 100 x 4.6 mm I.D., eluent; 0.1% formic acid / 0.1% formic acid -acetonitrile (70/30), flow rate; 1.0 mL/min, detection; UV at 260 nm, temperature; 25°C, injection volume; 10 μ L.

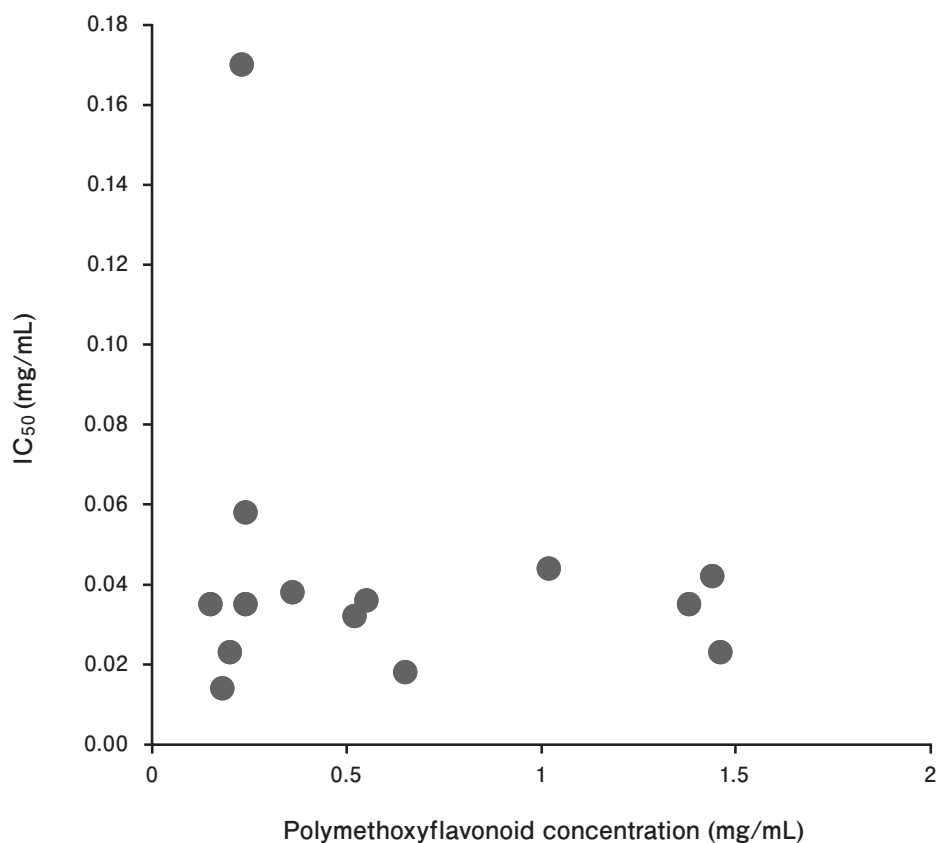


Fig. 3. Correlation of IC₅₀ to concentration of polymethoxyflavonoids.

The amount of polymethoxyflavonoids measured by HPLC method. IC₅₀ calculated HSA-glucose glycation model test result. IC₅₀; 50% inhibitory concentration of AGE formation; AGE, advanced glycation end product.

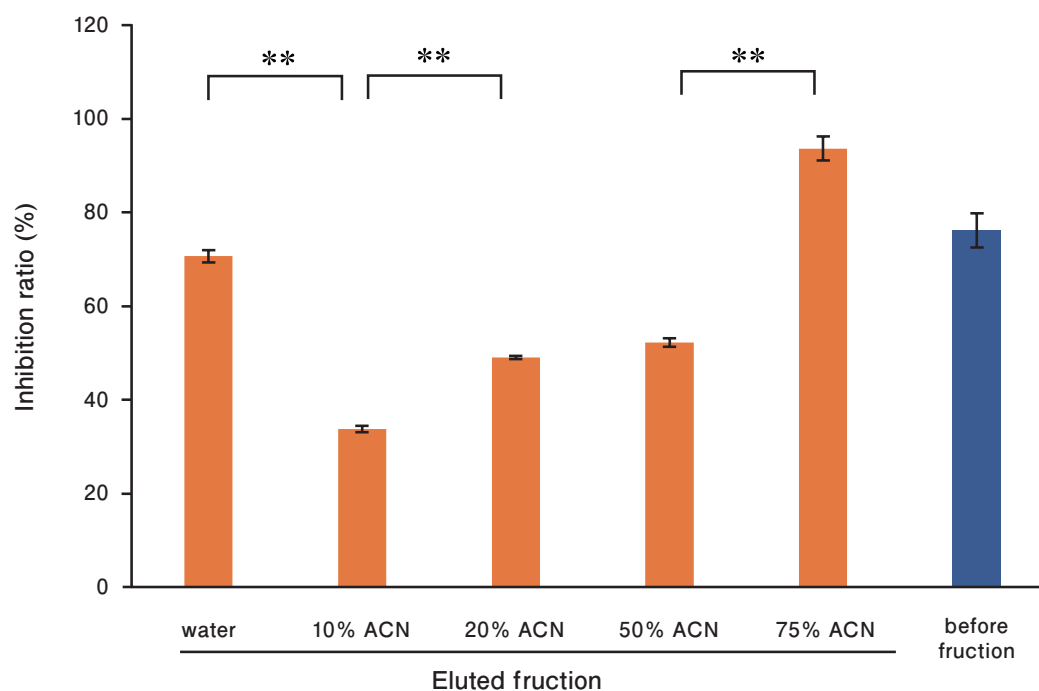


Fig. 4. Inhibition ratio of AGE formation to eluted fraction by the hydrophobic cartridge column.

Sample; hot-water extract of v216-1 (reference of Table 1). Hydrophobic cartridge column: Oasis HLB, Inhibition ratio of AGEs tested HSA-glucose glycation model, **, $p < 0.01$ by Tukey's test, ACN; acetonitrile; AGEs, advanced glycation end products; HSA, human serum albumin.

Discussion

PMF and Anti-glycation activity of KP

This study aimed to analyze active constituents of KP as an inhibitor on glycation, comparing, regarding inhibitory effectiveness, 10 types of extracts of KP rhizomes (4 types of dried powder and 6 types of extract powder), and examining the correlation between inhibitory effects on glycation and 5 types of PMF contained in KP.

The inhibitory effects on glycation tended to be stronger in hot-water extracts of dried powder than in 70% ethanol extract solutions and essence extract solutions of dried powder. The amount of PMF, which was contained in KP, was larger in 70% ethanol extracts than in hot-water extracts ($p < 0.03$). There was no recognized correlation between the IC_{50} of the KP sample solutions and content quantity of PMF. Moisture ethanol extract of KP has anti-glycation activity, as previously reported, and PMF is reported to be its active component¹⁹. This study confirmed that 4 types of PMF, excluding that of 3,5,7-trimethoxyflavone, had anti-glycation activities as well as involvement of PMF in the anti-glycation effect of KP. Furthermore, this study examined fractions which did not contain PMF (water elution), other than fractions which contained PMF (50% ACN and 75% ACN), regarded eluted fractions of hot-water extracts by the hydrophobic cartridge column, and clarified that anti-glycation activities were detected in the fractions without PMF (water elution). Therefore, it is assumed that components other than PMF would greatly contribute to anti-glycation activities of KP. Other than for PMF, there were two possibilities, at most, for components which have anti-glycation effects; one possibility was a hydrophilic substance which was maintained in the hydrophobic cartridge column, and the other was a substance which existed in the eluted fraction of 75% ACN in the hydrophobic cartridge column. The total amount of PMF (except for 3,5,7-trimethoxyflavone), which contained in solution eluted with fractionation using the hydrophobic cartridge column, was 0.266 mg/mL in 50% ACN and 0.245 mg/mL in 75% ACN. However, the inhibition concentration of the AGEs formation was 52.2% in 50% ACN and 93.6% in 75% ACN. That is, anti-glycation activities of eluted fractions did not depend on PMF concentrations. The reason that the inhibition ratio of 75% ACN was 1.8 times larger than that of 50% ACN may have been due to the possibility that the substance which should promote the glycation was removed through fractionation and purification. Thus, further examinations are required for verification on purification of eluates. It has been previously reported that KP rhizomes contain 11 types of flavonoid²² and phenolic glycosides²³. However, it has not been identified that these constituents would fulfil the role of anti-glycation activity.

Treatment process to utilize KP rhizomes

For utilization of KP as health foods, diversified treatment processes are performed. The test samples used in this study were dried products and essence extract powders of KP rhizomes which are sold or marketed. The KP rhizomes were for drying as slices (v220) or powder (v216-1, v216-2, v219). For methods for drying KP, low-temperature vacuum dehydration (v216-1) and high-temperature drying (v216-1, v216-2 and v219) were performed. In addition, hydrous ethanol extract method was employed to produce

essence powder (v217, v218, v221, v222, v223 and v224). Powder samples that were manufactured in high-temperature drying (v216-2 and v219) appeared black in color. Contrarily, powder samples with low-temperature dehydration (v216-1) appeared in a color of dark purple. It was assumed that thermal denaturation of anthocyanin contained in KP would affect the black color of the sample powder dried at a high temperature (v216-2 and v219) depending on the drying condition. Regarding the hot-water extract, the IC_{50} of the low-temperature vacuum dehydration (v216-1) was lower than the high-temperature drying (v216-2). Contrarily, regarding the 70% ethanol extract, the high-temperature drying (v216-2) was lower than the low-temperature vacuum dehydration (v216-1). It was assumed that the hydrophilic components of anti-glycation activities contained in the KP could be easily affected by heat.

KP contains PMF as a characteristic component. It has been reported that the KP have beneficial effects, such as improvement of metabolic syndrome, improvement of muscular metabolism dysfunction¹⁵, obesity amelioration¹⁶ and enhancement of physical strength^{17, 18}. As PMF is hydrophobic, ethanol is utilized to obtain PMF efficiently from KP. This study also indicated that larger amounts of PMF extract were obtained through 75% ethanol dried powder extraction and essence extraction rather than through hot-water extraction.

In the case where the content quantity of PMF was focused as the usage of KP, there was a possibility that 70% ethanol extraction of low-temperature dehydration would be advantageous. Contrarily, focusing on anti-glycation activity, there was a possibility that the hot-water extraction of the low-temperature dehydration powder would be advantageous. In the case where two activities must be covered, direct ingestion of dried powder was recognized to be a usage which has the least influence of extraction. It was suggested that proper treatment process would be required to suit its purpose.

Conclusion

This study compared 10 types of rhizomes of KP (*Kaempferia parviflora* Wall. Ex. Baker) and 5 types of PMF contained in KP regarding anti-glycation effects, and examined correlations between quantity of PMF and anti-glycation activities. There was no recognized correlation between content quantity of PMF and inhibitory effects of KP extract solutions. The examinations for anti-glycation effects employing the fractionation and purification of KP extracts suggested that active components, except for that of PMF, would exist as a hydrophilic substance. A possibility was indicated that some components greatly contributed to the anti-glycation activity of KP excluding PMF.

Statement of conflict of interest

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