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Original Article

Formulation of five curry spice mixtures and investigation of their effect on advanced glycation endproduct formation

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Abstract

***Objectives*:** Curry is a common food containing numerous spices known to have anti-oxidative or anti-glycative actions, which prevent advanced glycation endproduct (AGE) formation. In order to develop an anti-glycative ready-to-serve curry, we formulated five combinations of curry spice mixtures and investigated their effect on AGE formation.

***Methods*:** Test samples were five mixtures of spices to make curry. Hot water and 50% ethanol extractions were used and the polyphenol content was measured by the Folin-Ciocalteu method. Using the *in vitro* human serum albumin/glucose model, percent inhibition of AGE formation was evaluated by measuring fluorescent AGE, pentosidine, 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO). Fluorescent AGE was measured by fluorescence spectroscopy at excitation 370 nm and emission 440 nm. Pentosidine, 3-DG, GO, and MGO were measured by high performance liquid chromatography (HPLC).

***Results*:** This is the first report of formulating curry spice mixtures with both hot water and 50% ethanol extraction from the anti-glycation point of view. The inhibitory activity of 50% ethanol extracted curry formulation against fluorescent AGE, pentosidine, and reactive carbonyl species (3-DG, GO and MGO) was better than that of the hot water extracted formulation. The carbonyl trapping activity of curry mixtures is correlated with their polyphenol content.

***Conclusion*:** This study provides information on spice mixture formulation from the point of view of anti-glycation. Further study will be necessary to measure the color, taste, moisture and pH of curry prepared from the formulation to make it a ready to serve curry.

***KEY WORDS*:** advanced glycation endproducts (AGEs), pentosidine, 3-deoxyglucoson, glyoxal, methylglyoxal

Introduction

Glycation is the nonenzymatic reaction between a protein and a reducing sugar, such as glucose and fructose. The addition of reducing sugars to amine groups in proteins leads to the formation of a Schiff base, which rearranges to form a more stable Amadori product. Advanced glycation end products (AGEs) are formed after a series of complex reactions (*e.g;* oxidation, phosphorylation) [1].

Spices are common food adjuncts that have been used as flavoring, seasoning, and coloring agents, and even preservatives, throughout the world for thousands of years, particularly in Bangladesh, India, China, and many other southern Asian countries [2]. It has been hypothesized that the development of diabetes and the accumulation of AGEs may be reduced by intake of natural anti-oxidants through the diet [3]. Spices have been studied for their anti-oxidant properties for at least 50 years [4]. Spices, like fruits, vegetables, and medicinal herbs, are known to possess a variety of anti-oxidant effects and properties [5,6]. Phenolic compounds in these plant components are closely associated with their anti-oxidant activity [4]. They have been reported to offer diverse bioactivities, including anti-inflammatory, anti-allergic, anti-viral/anti-bacterial, anti-mutagenic/anti-carcinogenic properties, and protective effects against diverse diseases [7]. Many of these biologically significant functions are believed to be mediated by phenolics' free radical scavenging capability, which combats the oxidative stress induced by reactive oxygen species [8].

It has been reported that spices with anti-oxidative and radical scavenging activity were found to possess an *in vitro* anti-glycation activity [9,10]. The proposed anti-glycation mechanism includes scavenging of free radical and reactive carbonyl species [8]. Even though intensive studies on the bioactive compounds and anti-oxidant and anti-glycation

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activity of many spices have been carried out, the anti-glycation activity of spice mixtures remains to be elucidated.

Spices are fundamental to all curry recipes and there is a huge range of spices, which are used to prepare curry. The spices are used to flavor the food, making each dish distinct and wonderfully aromatic. Each spice by itself imparts a very unique flavor, but when used together with other spices, the combination and permutation of different spices magically change the individual characteristics. Curry is a dish with origins in South and Southeast Asians cuisines. The common feature is the incorporation of complex combinations of spices or herbs, usually including fresh or dried hot chilies. Some limit the use of the term "curry" to dishes prepared in a sauce [11,12], but curries may be wet or dry. The main spices found in most South Asian curry powder are chili, onion, turmeric, ginger, cinnamon, bay leaves, and cloves. A wide range of additional spices may be included depending on the geographic region and the foods which are added, such as white/red meat, fish, lentils, rice and vegetables [13].

In this regard, the present study attempted to formulate five combinations of curry spice mixtures, investigates their total phenolic contents, and evaluate their effect on fluorescent AGEs and some AGEs representatives such as 3-DG, GO, MGO and pentosidine, with the aim to develop an anti-glycative ready-to-serve curry.

Materials and Methods

Preparation of spice mixture extract

Test components were curry spice mixtures, of which the components are listed in ***Table 1***. The spice mixture samples were dried at 65ºC for 72 hours and ground into fine powders. Two grams of each type of spice power was extracted with 20 mL of 100% distilled water at 80ºC in a water bath for one hour. Spice power was also extracted with 20 mL of 50% ethanol at 49ºC in a water bath for 4 hours. The concentration of each sample was estimated from the weight difference before and after incubation of 5 mL sub-samples, dried in aluminum trays at 120°C for 1.5 hours. The extracts were kept at - 20°C until investigation of their effect on the generation of fluorescent AGEs, formation of AGE intermediates (3-DG, GO and MGO) and pentosidine in the human serum albumin (HSA)/glucose model [1].

In vitro HSA/glucose reaction model

The glycation of HSA and formation of AGEs was modeled by incubating HSA with and without glucose at 60°C for 40 hours as previously reported [1]. The glucose (+) reaction solution contained 0.1 M phosphate buffer (pH 7.4), 40 mg/mL HSA, 2.0 M glucose solution, and distilled water at a 5:2:1:2 volume ratio. The glucose (-) reaction solution contained 0.1 M phosphate buffer (pH7.4), 40 mg/mL HSA, and distilled water at a 5:2:3 volume ratio.

Measurement of fluorescent AGEs

AGE-derived fluorescence was measured as reported previously using an *in vitro* HSA/glucose model [1,14]. Briefly, 100 µL of various concentrations of test samples in aqueous solution were added to 500 µL of 0.1 mol/L phosphate buffer solution (PBS, pH 7.4), 100 µL distilled water, 200 µL 40 mg/mL HSA (Sigma Chemical Co., Ltd., St. Louis, MO, USA), and 100 µL 2.0 mol/L aqueous solution of glucose. Distilled water was then added to make up a total volume of 1.0 mL, and the material was incubated at 60°C for 40 hours (Solution A). Final concentrations were 8 mg/mL HSA and 0.2 mol/L glucose. At the same time, a solution including distilled water, added in lieu of aqueous glucose, was incubated as a blank for each reaction (Solution B). Samples prepared without the addition of test samples were incubated as a positive control (Solution C). And at the same time, a solution including distilled water, added in lieu of aqueous glucose, was incubated as a blank for positive controls (Solution D). Fluorescent AGEs were measured quantitatively in each sample reaction solution (A, B, C, D) to evaluate inhibitory activity on AGE formation. Fluorescent AGEs were measured using an ARVO MX 1420 ARVO series Multilabel Counter (Perkin-Elmer Japan Corp, Yokohama, Japan) microplate reader at an excitation wavelength of 370 nm and a

Table 1. Component of curry spice mixture.

Spice	Category-I (%)	Category-II (%)	Category-III (%)	Category-IV (%)	Category-V (%)
Red chilli	40	20	30	15	10
Onion	20	30	20	30	20
Turmeric	15	15	10	10	15
Ginger	10	20	10	20	30
Garlic	10	5	5	2	3
Cinnamon			5	4	2
Cardamom					2
Bay leaf	5		5	5	2
Cloves		10	5	5	5
Cumin			5	6	10
Coriander			5	3	1

fluorescence wavelength of 440 nm. Calibration curves for inhibition of fluorescent AGE formation (%) were constructed by adding individual samples to a reaction solution at three concentrations (0.1%, 0.01%, 0.001%) and calculating the inhibition of fluorescent AGE formation (%) after the reaction. The measurement was repeated three times for each sample.

Measurement of 3-DG, GO and MGO

For AGE intermediates, 3-DG, GO and MGO were measured by HPLC (LC-20AT system equipped with SPD-20A UV/VIS detector and LC-solution software; Shimadzu, Kyoto, Japan). Briefly, 10 μg/mL 3-DG, GO, and MGO standard solution was prepared by mixing 0.4 mL of 50 μg/mL 3-DG, 0.4 mL of 50 μg/mL GO and 0.4 mL of 50 μg/mL MGO with 1.2 mL of distilled water. Standard solutions of 1.0, 0.25, 0.05, 0.025, and 0 μg/mL were prepared by serial dilutions with distilled water from 10 μg/mL. Then, 200 μL standard solution, 100 μL of 200 mM phosphate buffer and 30 μL of distilled water were added to a microtube and mixed properly; 200 μL of antiglycation test reagent with/without spice sample were added to 130 μL of distilled water in a microtube and mixed properly; 170 μL of 6% perchloric acid was added separately to both the sample and standard solutions, and centrifuged at 12,000 rpm for 10 minutes. After centrifugation 400 μL of supernatant was transferred into another microtube. This was followed with the addition of 350 μL saturated sodium bicarbonate, and finally 50 μL of 2, 3- diaminonaphthalene as an internal standard. After mixing, the reaction mixtures were allowed to incubate at 4ºC for 24 hours. After incubation, the reaction mixtures were centrifuged at 15,000 rpm for 10 minutes, and the supernatant was used for analysis by HPLC-UV. The analytical conditions were as follows: eluent 50mM phosphate : acetonitrile (ACN) = 89:11, YMC-pack CN, S-5 μm, 150 × 4.6 mm ID (YMC, Kyoto, Japan), a flow rate of 1.0 mL/min, column temperature 40ºC, detection wavelength 268 nm, injection volume 20 μL. A 3-DG, GO and MGO standard curve was constructed by measuring peak areas of the standard solutions. The 3-DG, GO and MGO inhibitory effect of each spice sample was determined from the standard curve by measuring the peak areas of the respective samples. Two measurements (n = 2) were taken for each sample.

Measurement of pentosidine

200 mL and 100 mL of extra for elute I and elute II respectively were prepared (***Table 2***). The evaporated residue was dissolved in 300 μL of elute-I, mixed by vortex, centrifuged and then transferred into HPLC vials. Pentosidine was measured by HPLC (LC-20AT system equipped with FS-8020 fluorescent spectrometer and LC-solution software; Shimadzu). The HPLC analytical conditions were as follows: separation column: YMC Triat C18 (column size 150 × 4.6 mm ID, 5 μm/12 nm; YMC); flow rate: 1.0 mL/min; and column temperature: 30ºC. The samples were detected using an excitation wavelength of 335 nm and a detection wavelength of 385 nm. Elution was performed using eluent I (ACN/Methanol/Water/Perfluobutyric acid [HFBA] = 16/4/76/0.2) and eluent II (ACN/HFBA = 100/0.2), and the following gradient conditions: analysis: 0 ~ 20 min with 0% eluent II; washing: 20 ~ 25 min with 100% eluent II; and conditioning: 25 ~ 40 min with 0% eluent II. A standard curve was generated using standard pentosidine samples. Three measurements (n = 3) were taken for each sample.

Measurement of polyphenols

As previously reported [15], the polyphenol content in each test sample was measured using the Folin-Ciocalteu (FC) method in conjunction with the "Commercial Product Test Results for Polyphenol-Containing Foods" produced by the National Consumer Affairs Center of Japan (NCAC). Specifically, 100 μL of test sample solutions was added to 50 μL of a 2-fold dilution of FC reagent (Wako Pure Chemical Industries) and 500 μL of 0.4 M aqueous Na_2CO_3 to make up a total volume of 650 μL; the material was left to stand at ambient temperature for 30 minutes, and absorbency at 660 nm was then measured. A (+)-catechin solution (Wako Pure Chemical Industries) was used as a standard. Multiple (+)-catechin solutions were prepared in 11 steps as 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.0125, 0.01, 0.00625, 0.001, and 0 mg/mL concentrations; 100 μL of each concentration of (+)-catechin solution was added to 50 μL of the 2-fold diluted FC reagent and 500 μL of 0.4 M aqueous Na_2CO_3 to make up a total volume of 650 μL, the material was left to stand at ambient temperature for 30 minutes, and absorbency at 660 nm was then measured. Using the (+)-catechin calibration curve prepared, total polyphenol content per solid unit of each test sample solution was calculated as a catechin equivalent (mg catechin Eq/mg solid content). Measurement was repeated three times for each sample.

Results

Effect of spice mixture on fluorescent AGE formation

Fig. 1. shows the inhibitory effects of five curry spice mixture formulations on the fluorescent AGE formation in the *in vitro* HSA/glucose model. Each sample showed actions of preventing fluorescent AGE formation. In the hot water extracts, mean values of % inhibition varied from 56.5% to 157.5%, while in the 50% ethanol extracts, values were stable between 84.6% and 91.6%.

Table 2. Component of elute - I and - II

Reagent	Elute-I	Elute-II (ratio)
Acetonitrile (ACN)	16	100
Methanol	4	–
Distilled water	76	–
Perfluoro butyric acid (HFBA)	0.2	0.2

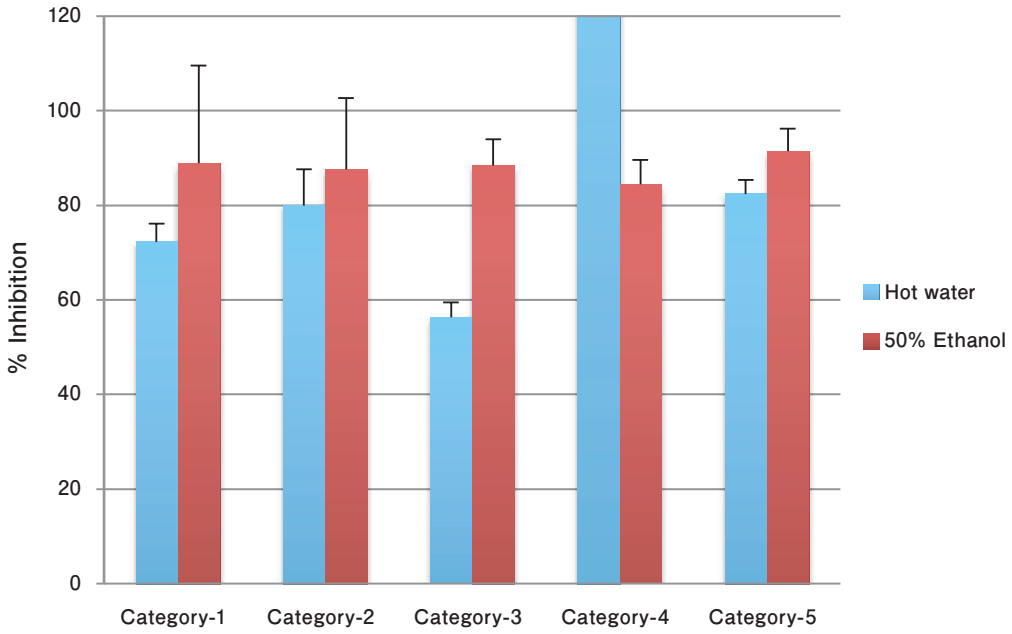


Fig. 1. **Inhibitory effects of curry spice mixtures on the formation of fluorescent AGEs in HSA/glucose model.**

Results are expressed as means ± standard deviation, n = 3. AGEs, advanced glycation endproducts; HSA, human serum albumin.

Effect of spice mixture on 3-DG, GO and MGO formation

Results of the effect of spice mixture on 3-DG, GO and MGO formation are presented in ***Figs. 2*** and ***3***. The hot water extracts showed inhibitory effects, except for Category-4 on the formation of MGO (70.4% - 82.0%) and GO (56.8% - 62.9%). The effect of 3-DG was low (12.9% - 36.6%).

All of the samples of the 50% ethanol extract showed inhibition of formation of GO (64.6% - 67.8%) and 3-DG (39.1% - 54.2%). The 50% ethanol extract from Category-4 showed no inhibition of MGO formation, however, in the rest of samples, % inhibition was from 42.0% to 54.8%.

Effect of spice mixture on pentosidine formation

The results of the effect of spice mixture on pentosidine formation are presented in ***Fig. 4***. The category-I sample showed marked inhibition on pentosidine formation in the *in vitro* HSA/glucose model. The % inhibition value reached almost 100% in the hot water extract and was 59.4% in 50% ethanol extract. In the other category samples, % inhibition was less than 32.2%. The values varied depending on the category.

Polyphenol content

Total polyphenol contents in each sample are presented in ***Fig. 5***. Values varied from 0.07 mg cathechin Eq/mL to 0.09 mg cathechin Eq/mL in hot water extracts and from 0.08 mg cathechin Eq/mL to 0.12 mg cathechin Eq/mL in 50% ethanol extracts. Polyphenol content tended to be higher in 50% ethanol extract than in hot water extract.

Discussion

Effect of spice mixture on fluorescent AGE formation

The HSA/glucose model adopted in this study provides a useful tool for assessing the effects of curry spice mixture on the formation of fluorescent AGEs with both hot water and 50% ethanol extractions. The first study displays the inhibitory effects of five curry spice mixture formulations on the fluorescent AGE formation in this model. The results revealed that 50% ethanol-extracted curry mixture showed higher fluorescent AGE inhibition activity than water-extracted curry mixture. The 50% ethanol extracts of all curry spice mixtures examined showed significant inhibitory activities above 90% on the generation of fluorescence AGE.

Relationship between total polyphenol contents and anti-glycation activities of curry spice mixture

Recently, various phenolic anti-oxidants from plant extracts have been found to inhibit the formation of AGEs [14,15], and their inhibition of free radical generation in the glycation process and subsequent inhibition of the modification of proteins have been considered as the major mechanisms which mediate their anti-glycative activities. Thus, in the following experiments, total polyphenol contents of the five curry spice mixtures with both water and 50% ethanol extraction were determined using a calorimetric method. It was found that 50% ethanol-extracted spice mixtures had higher polyphenol contents than water-extracted spice mixtures. Anti-glycative activities of 50% ethanol-extracted spice mixtures were correlated with their total polyphenol contents ($R^2 = 0.188$) (***Fig. 6***), suggesting that the phenolic ingredients play a major role in the anti-glycative activities of spice mixtures.

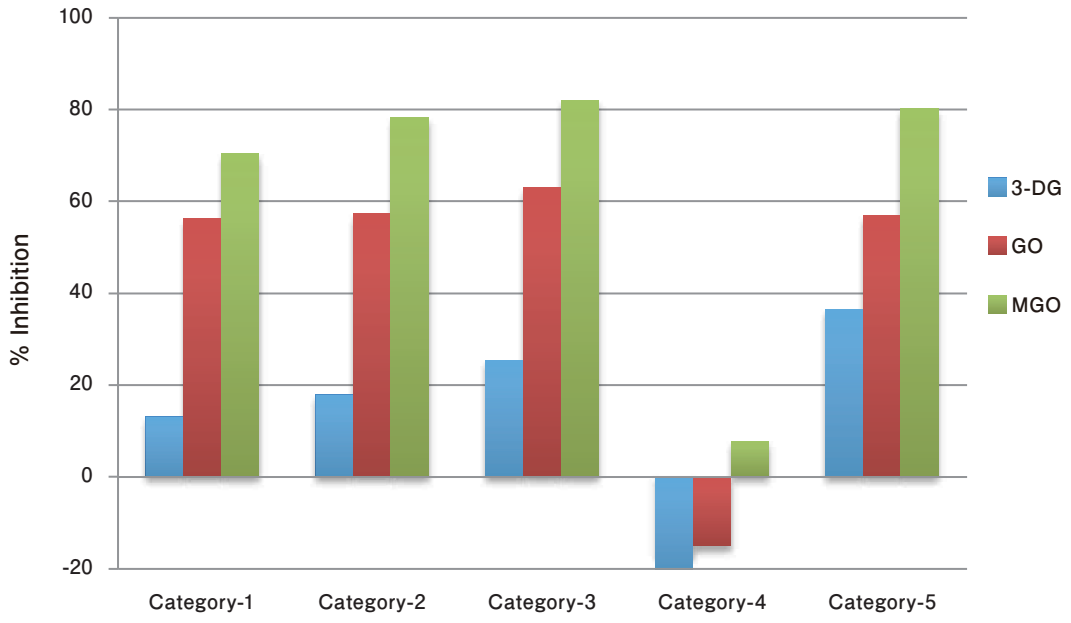


Fig. 2. **Effects of hot water extracts from curry spice mixtures on the formation of 3-DG, GO, and MGO.**

In vitro HSA/glucose model. Results are expressed as means of duplicate measurements. HSA, human serum albumin; 3-DG, 3-deoxyglucosone; GO, glyoxal; MGO, methylglyoxal.

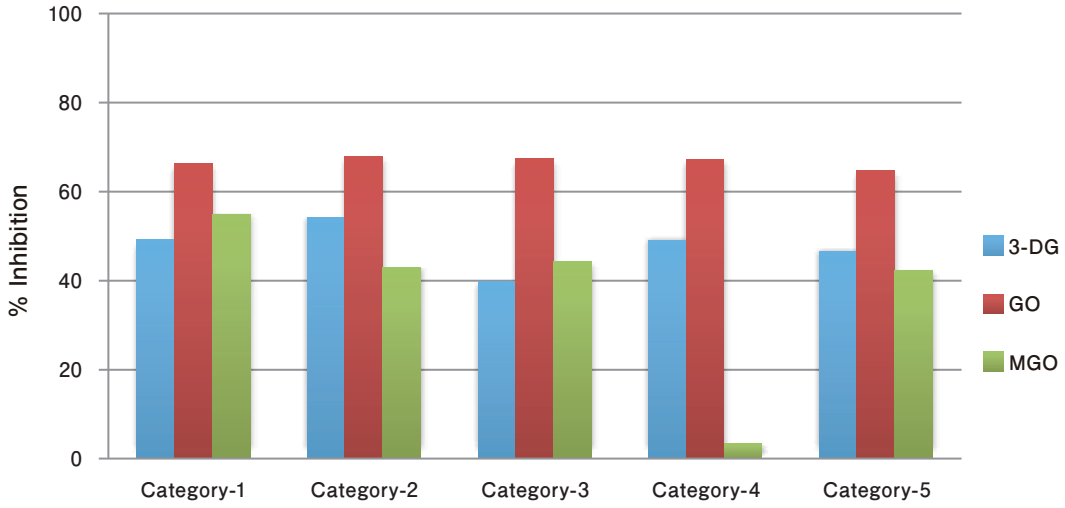


Fig. 3. **Effects of 50% ethanol extracts from curry spice mixtures on the formation of 3-DG, GO, and MGO.**

In vitro HSA/glucose reaction model. Results are expressed as means of duplicate measurements. HSA, human serum albumin; 3-DG, 3-deoxyglucosone; GO, glyoxal; MGO, methylglyoxal.

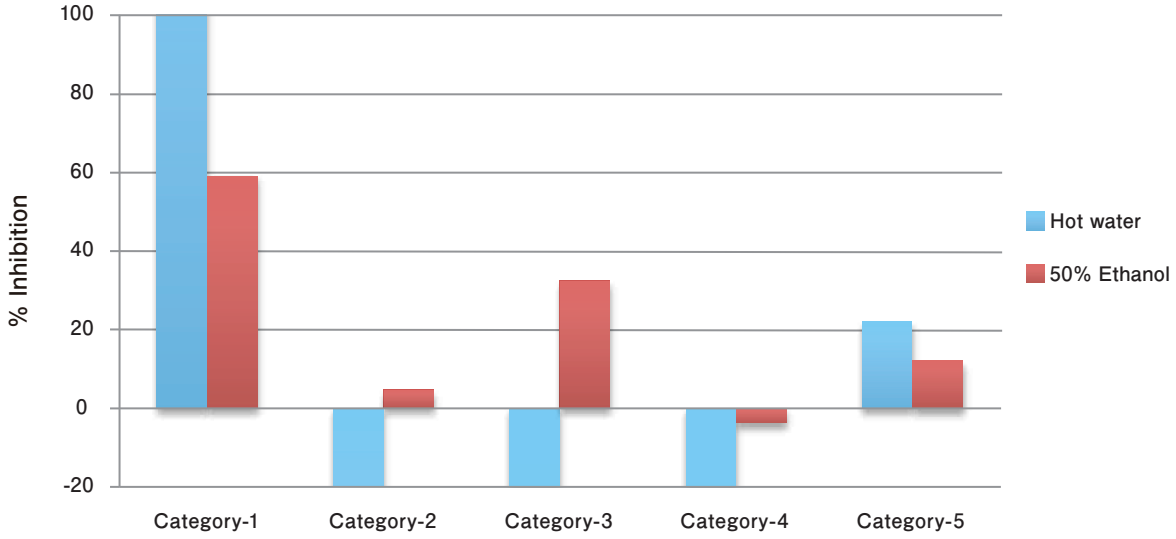


Fig. 4. **Effect of curry spice mixtures on the formation of pentosidine in the *in vitro* HSA/glucose model.**
Results are expressed as means of duplicate measurements. HSA, human serum albumin.

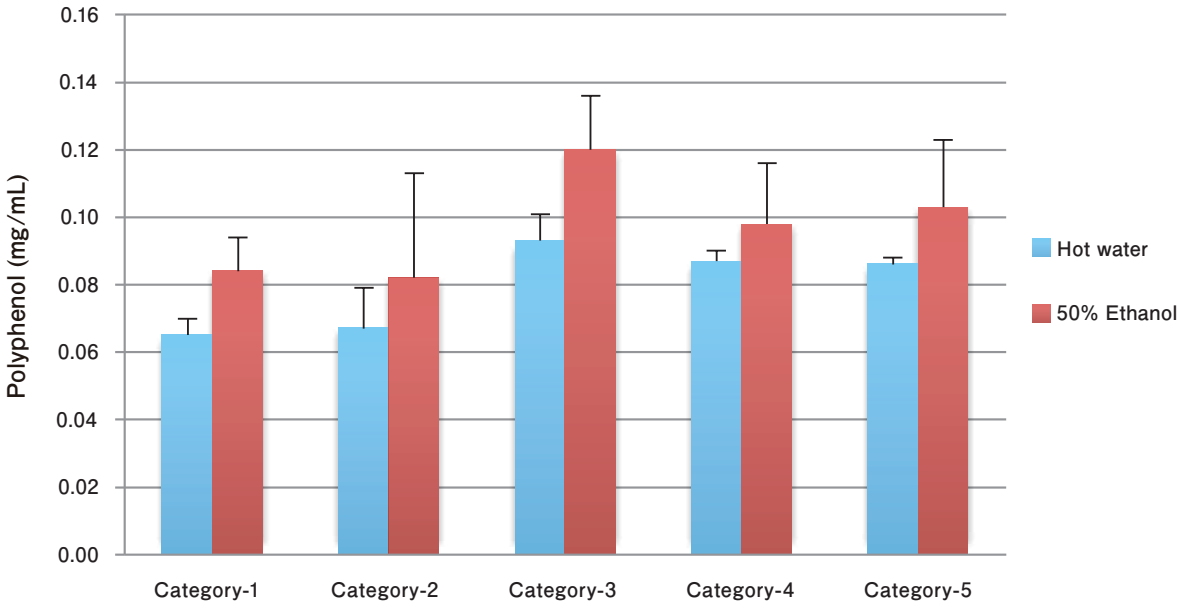
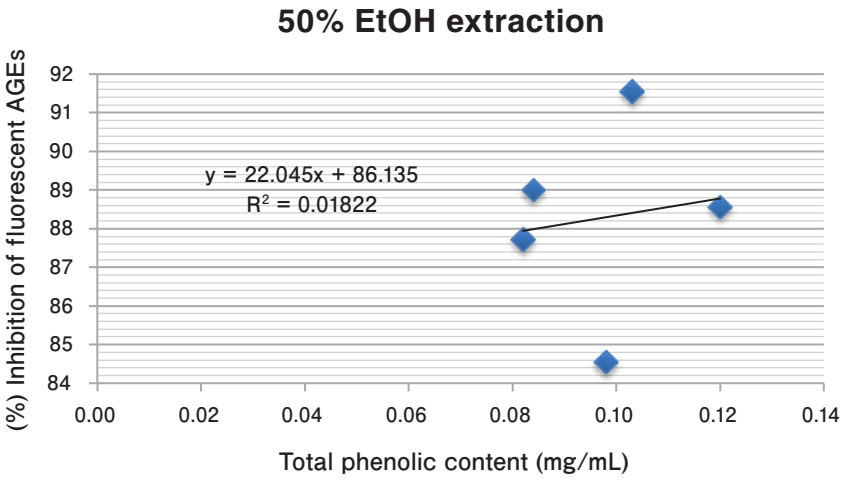


Fig. 5. **Total polyphenol contents of both hot water and 50% ethanol extracted from five curry spice mixtures.**
Results are expressed means ± standard deviation, n = 3.

a)



b)

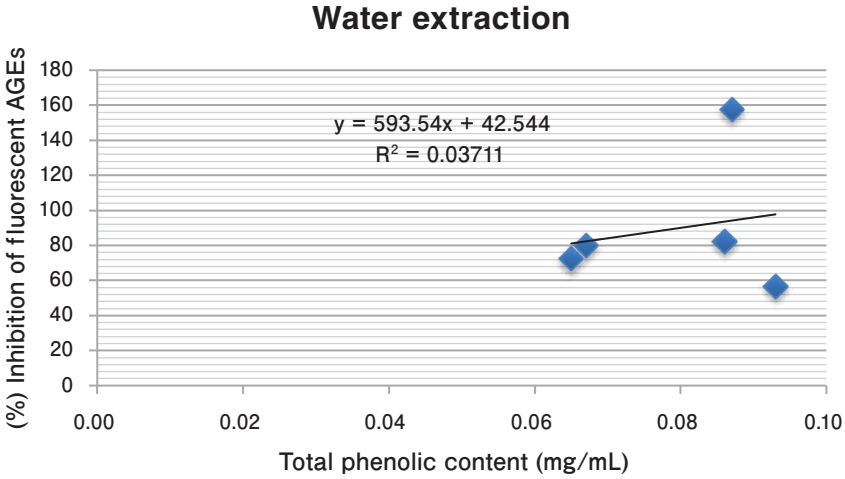


Fig. 6. **Linear relationship between % inhibition of fluorescence AGE formation and total polyphenol contents of five curry spice formulations.**

a) 50% ethanol extraction, b) Hot water extraction.

Inhibitory effects of curry spice mixtures on the formation of 3-DG, GO and MGO

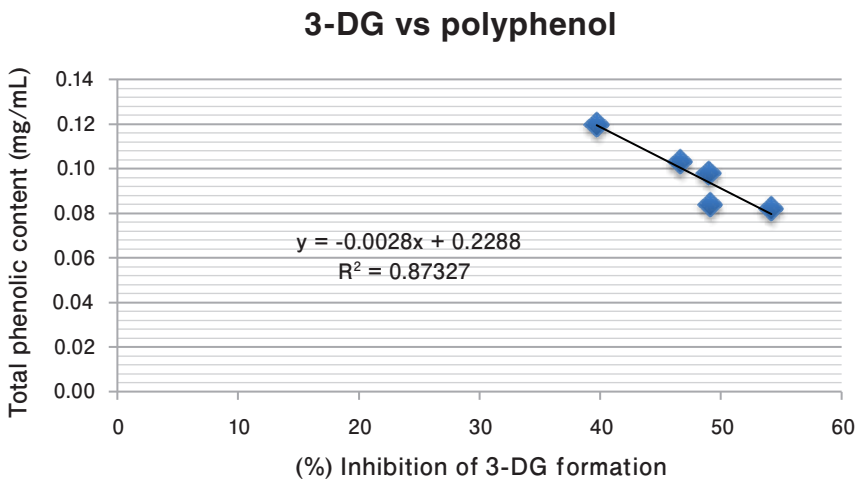
Several aldehydes, such as 3-DG [16], GO [17], and MGO [16] are generated in the Maillard reaction and contribute further to the formation of AGEs.

All curry spice mixtures with 50% ethanol extraction exhibited strong inhibitory effects on the formation of 3-DG, GO, and MGO. Mixture category-1 exhibited the strongest inhibition with 49.1% for 3-DG, 66.1% for GO, and 54.8% for MGO. It is interesting to note that the 50% ethanol-extracted curry spice mixture predominantly inhibited the formation of GO (nearly 66%). Correlative analysis revealed that 3-DG inhibition activity is highly correlated with the polyphenol content ($R^2 = 0.870$) (***Fig. 7-a***). MGO inhibition activity was poorly correlated with the polyphenol content ($R^2 = 0.160$) (***Fig. 7-c***), and GO inhibition was not correlated with polyphenol content ($R^2 = 0.015$) (***Fig. 7-b***). We predict that the higher carbonyl trapping activity of the 50% ethanol-extracted spice mixture is brought about by both the hydrophobic and hydrophilic phenolic compounds.

As seen in ***Fig. 6***. the carbonyl trapping activity of the water-extracted curry spice mixture is comparatively lower than the 50% ethanol-extracted mixture. Among the water-extracted mixture category-3 exhibited the highest inhibition with 25.4% for 3-DG, 63.0% for GO, and 82.0% for MGO. It is noteworthy that the curry spice mixture primarily inhibited the formation of MGO with water extraction. It was previously reported that water-soluble cathechin, epicatechin, and procyanidin B2 from cinnamon more markedly inhibit methylglyoxal (about 93%) than aminoguanidin (73%) [18]. We assume that the higher MGO inhibitory effects of water-extracted curry spice mixture is brought about by the water soluble polyphenols, such as catechin, epicatechin, and procyanidin B2 from cinnamon, cloves, bay leaf, and turmeric. Correlative analysis showed that the carbonyl-trapping activity of water-extracted curry spice mixture was not correlated with the polyphenol content (data not shown).

A 50% ethanol extracted curry spice mixture is best suited to prepare the real curry. In real curry oil, ghee as a clarified butter, groundnut oil, and sunflower oil with water are used as the medium to carry the spices, which makes the curry a suitable environment for hydrophobic and hydrophilic components in spices.

a)



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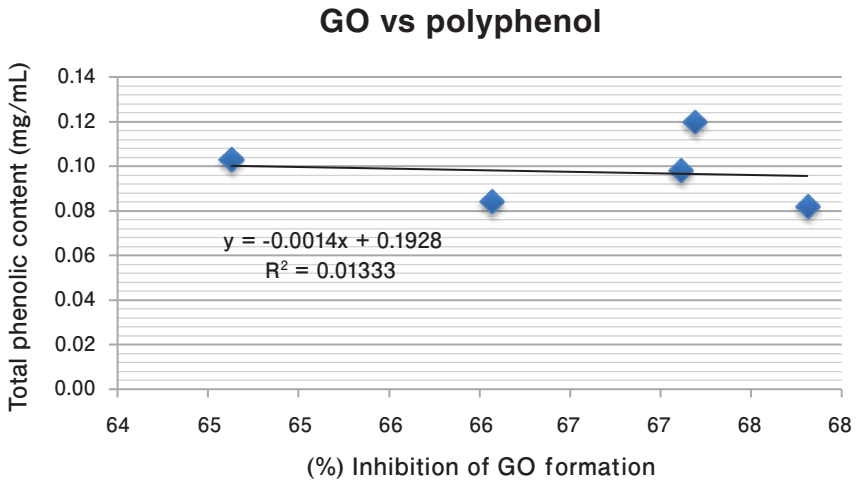

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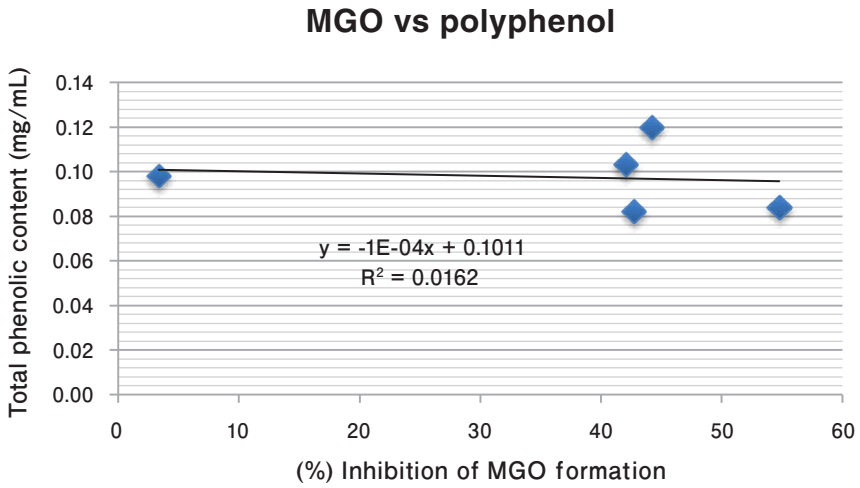

Fig. 7. **Relationship between: (a), % Inhibition of 3-DG formation and total polyphenol content; (b), % Inhibition of GO formation and total polyphenol content; (c), % Inhibition of MGO formation and total polyphenol content of 50% ethanol extracted curry spice mixtures.**

3-DG, 3-deoxyglucosone; GO, glyoxal; MGO, methylglyoxal.

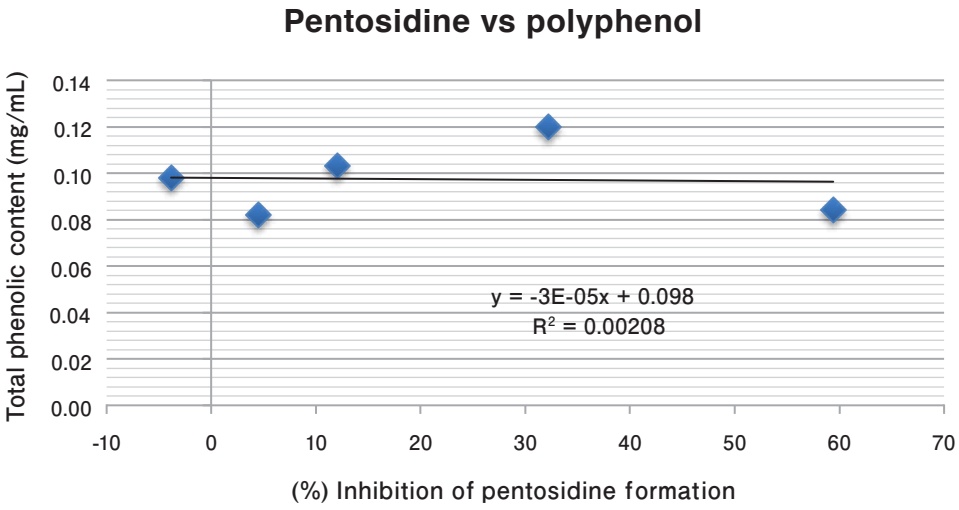

Fig. 8. **Relation between (%) inhibition of pentosidine formation and total polyphenol content.**

No correlation between (%) inhibition of pentosidine formation and total polyphenol content (mg catechin Eq/mL).

Inhibitory effects of curry spice mixture on pentosidine formation

The 50% ethanol extracted curry spice mixtures exhibited pentosidine inhibitory effect except for Category-4 (***Fig. 4***). Among the water-extracted curry spice mixtures, only Category-5 exhibited an effect on pentosidine formation. It has been reported that 3-DG, fructose, ascorbate, and Amadori compounds act as precursors for the formation of pentosidine [1,19]. As seen from ***Fig. 2***, among the water-extracted spice categories, the 3-DG inhibitory effect of Category-5 was higher. This high 3-DG inhibitory effect of Category-5 might affect the pentosidine formation. The inhibitory effects of 50% ethanol-extracted spice categories on pentosidine formation are 59.4% for Category-1 and 32.2% for Category-3. ***Figure 8*** showed that pentosidine inhibitory effect of 50% ethanol-extracted curry spice mixtures was not correlated with their polyphenol contents.

On the basis of the anti-glycation, and anti-oxidant activity, and polyphenol content, we assume that the anti-glycation activity of spice mixtures is contributed by the anti-oxidant activity of cinnamon, ginger, cloves, bay leaf, and turmeric. It has been reported that boiling and steaming causes significant decreases in phenolic content and anti-oxidant power [20]. Peng *et al*. reported that although fortification of bread with grape seed extract (GSE) enhances the anti-oxidant capacity, baking leads to a 30 - 40% decrease in GSE's anti-oxidant capacity [21]. An increase in anti-oxidant activity with thermal processing has also been observed in studies of pressure-streaming yellow beans, cooking tomato, and high-pressure processing of tomato and carrot purees; this phenomenon was correlated with an increase of particular phenolic contents [20,22,23]. It is possible to prepare a better curry from the viewpoint of anti-glycation with vegetables, such as tomatoes, carrots, and beans, those by which the anti-oxidant activities increase with thermal processing.

Conclusion

This is the first report of formulating curry spice mixtures from the viewpoint of anti-glycation. The present study revealed that 50% ethanol-extracted curry spices mixtures showed a higher fluorescent AGE inhibition, and carbonyl trapping inhibition activity than hot water-extracted curry spice mixtures. The fluorescent AGE inhibition activity and carbonyl trapping activity of 50% ethanol-extracted curry spice mixtures are correlated with their polyphenol content. Further investigation will be necessary to prepare curry from our formulations to achieve ready-to-serve curry to combat glycative stress.

Acknowledgements

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Conflict of Interest Statement

The authors state that performance of this study entailed no issues representing a conflict of interest.

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