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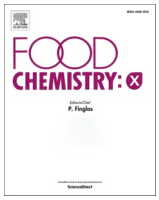

Novel inhibitory effect of black chokeberry (*Aronia melanocarpa*) from selected eight berries extracts on advanced glycation end-products formation and corresponding mechanism study

Hui Tan [a], Baoyue Cui [a], Kexin Zheng [a], Ningxuan Gao [a], Xuening An [a], Yu Zhang [a], Zhen Cheng [a], Yujie Nie [a], Jinyan Zhu [b], Li Wang [d], Kuniyoshi Shimizu [c], Xiyun Sun [a,*], Bin Li [a,*]

[a] College of Food Science, Shenyang Agriculture University, Shenyang, Liaoning 110866, China
[b] Zhuanghe Food Inspection and Monitoring Center, Dalian, Liaoning 116400, China
[c] Department of Agro-Environmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
[d] Liaoning Lingxiu Mountain Shenghui Industrial Group Co. LTD, Liaoyang, Liaoning Province 111008, China

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ABSTRACT

Numerous health hazards have been connected to advanced glycation end products (AGEs). In this investigation, using reaction models including BSA-fructose, BSA- methylglyoxal (MGO), and BSA-glyoxal (GO), we examined the anti-glycation potential of eight different berry species on AGEs formation. Our results indicate that black chokeberry (*Aronia melanocarpa*) exhibited the highest inhibitory effects, with IC_{50} values of 0.35 ± 0.02, 0.45 ± 0.03, and 0.48 ± 0.11 mg/mL, respectively. Furthermore, our findings suggest that black chokeberry inhibits AGE formation by binding to BSA, which alleviates the conformation alteration, prevents protein cross-linking, and traps reactive α-dicarbonyls to form adducts. Notably, three major polyphenols, including cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, and procyanidin B2 from black chokeberry, showed remarkably inhibitory effect on MGO/GO capture, and new adducts formation was verified through LC-MS/MS analysis. In summary, our research provides a theoretical basis for the use of berries, particularly black chokeberry, as natural functional food components with potential anti-glycation effects.

Introduction

Glycation reactions, often referred to as non-enzymatic glycation reactions, are non-enzymatic condensation reactions involving the free amino group on macromolecules like protein, lipid, or nucleic acid with the carbonyl group on reducing sugar. Advanced glycation end-products (AGEs) are persistent covalent adducts that result from these processes. Three phases may often be identified in the production of AGEs. The amino group first engages in nucleophilic addition with the carbonyl group to produce a reversible Schiff base, which then undergoes rearrangement to produce stable Amadori products (ARPs). In the intermediate step, oxidative fission or *retro*-aldol fragmentation of ARPs produces α-dicarbonyls, such as methylglyoxal (MGO), and glyoxal (GO). These substances are ultimately converted into dark brown cross-linked polymeric melanoidins (Dariya, & Nagaraju, 2020; Jia, Ma, Zhang, Fan, & Shi, 2022; Chen, Yen, Tsai, & Lin, 2021). AGEs can be exogenous (derived from food) or synthesized endogenously in the body. Studies have revealed that the accumulation of endogenous or exogenous AGEs is a key factor in the emergence of chronic age-related diseases, such as Alzheimer's disease, diabetes, cardiovascular disease, and nervous system disorders (Basta, Schmidt, & De Caterina, 2004; Kuhla et al., 2007). According to earlier study, aminoguanidine (AG) is an effective anti-glycation agent(Okamoto, & Okabe, 2000; Reddy, & Beyaz, 2006).However, long-term intake of AG may have certain side effects, such as delaying cell proliferation, affecting renal function, and increasing the risk of cancer(Wang et al., 2021).Therefore, there is considerable interest in finding novel phytochemicals from dietary vegetables, fruits, and herbal plants that safely and effectively prevent the production of AGEs.

It is well known that berries include high concentrations of phenolic compounds, such as phenolic acids, flavonoids (including anthocyanins, flavonols, and catechins), and tannins (procyanidins found in black-colored berries). Phenolic compounds, due to their special molecular structure, have been demonstrated to have inhibitory effects on AGEs

* Corresponding authors.
E-mail addresses: sun_xiyun@163.com (X. Sun), libinsyau@163.com (B. Li).

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formation through several mechanisms, including reducing the generation of ARPs, scavenging free radicals, the creation of phenolics-MGO/GO adducts to trap reactive carbonyl species, and the modification of protein glycation sites (Velichkova, Foubert, & Pieters, 2021). Previous studies have demonstrated that extracts from blueberry fruits significantly inhibit AGEs formation, their phenolic components, such as catechin, which scavenge methylglyoxal by forming adducts with them, were thought to be responsible for the antiglycation properties (Wang, Yagiz, Buran, Nunes, & Gu, 2011).Additionally, it has been demonstrated that a phenolic composition from Chinese bayberry can suppress the glycation reaction by scavenging free radicals, chelating metal ions, and trapping carbonyl compounds to reduce the production of AGEs (Zhang et al., 2021). However, because the phenolic content and bioactive composition of various berry fruits can differ, it is important to systematically compare the effectiveness of various berry species in reducing AGEs in relation to their chemical compositions, particularly during both the early and late stages of glycation (Zhao et al., 2022).

The aim of this study was to evaluate the anti-glycation capacity of novel candidates from berries and define their inhibition mechanism (Sarmah, & Roy, 2022). We comprehensively studied and compared eight different varieties of berries on AGEs formation, including blueberry (*Vaccinium corymbosum*), strawberry (*Fragaria* × *ananassa Duch.*), raspberry (*Rubus corchorifolius*), cranberry (*Vaccinium Oxycoccos*), black chokeberry (*Aronia melanocarpa*), kiwi berry (*Actinidia arguta*), sea buckthorn (*Hippophae rhamnoides*), and blackcurrant (*Ribes nigrum L.*). After preliminary screening for anti-glycation effects, we further investigated the mechanisms of the active extracts and their constituents by studying their abilities to scavenge free radicals, prevent protein glycation and crosslinking, and trap reactive α-dicarbonyls to form adducts. We aim to contribute to the development of new berry resources as potential food ingredients with anti-glycation effects by providing a reference for future studies.

Materials and methods

Chemicals

BSA (purity > 96 %) was acquired from YuanYe Biotechnology Co., Ltd. (Shanghai, China). D-fructose, AG, methylglyoxal (MGO, 40 % v/v) and glyoxal (GO, 40 % v/v) were obtained from Solarbio Technology Co., Ltd. (Beijing, China). Cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-galactoside and procyanidin B2 were purchased from ChemFaces Biochemical Co., Ltd. (Wuhan, China). o-Phenylenediamine (OPD), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and other chemical reagents were bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), analytical grade.

Preparation of berry extracts

Blueberry (*Vaccinium corymbosum*) was provided by Shenyang Qipanshan *Gem* Crown Blueberry Industry Co., Ltd. (Shenyang, China). Strawberry (*Fragaria* × *ananassa Duch.*) was provided by Dandong YiNong Food Co., Ltd. (Dandong, China). Raspberry (*Rubus corchorifolius*), cranberry (*Vaccinium Oxycoccos*), black chokeberry (*Aronia melanocarpa*), sea buckthorn (*Hippophae rhamnoides*), kiwi berry (*Actinidia arguta*) and blackcurrant (*Ribes nigrum L.*) were provided by Dandong JunAo Food Co., Ltd. (Dandong, China).

The berries were extracted using an aqueous solution of 70 % ethanol (v/v) at 45℃ for 90 min. The supernatant was transferred after the extract was centrifuged at 5000 rpm for 20 min. The precipitate was re-extracted three more times as previously indicated, and then the supernatants were combined, filtered, and then evaporated at 45℃ under reduced pressure. Prior to further examination, the concentrated solutions were lyophilized and stored at −80℃.

Chemical analyses of berry extracts

According to our prior publication, the Folin–Ciocalteau method was used to determine the total phenolic content (TPC) of each sample of berry (Wang et al., 2016). Gallic acid was used as the standard substance for creation of a standard curve and results were expressed in mg of gallic acid equivalent per gram of dried extract (mg gallic acid equiv./g DW)(Li et al., 2017). Total flavonoid content (TFC) was determined using sodium nitrite-aluminum nitrate colorimetry with slight adjustments (Liu et al., 2022; Wang, Gao, Li, Tan, & Zheng, 2019). Rutin concentration was used as a standard for TFC determination. The total anthocyanins content (TAC) was determined utilizing a pH-differentiated procedure, which was then expressed as mg cyanidin-3-glucoside (C3G) equivalents per liter (Wang et al., 2016). The butanol-HCl test was slightly modified to assess the total procyanidins content (TPAC), and procyanidins B2 served as the standard (Gao et al., 2023).

Anti-glycation assay in BSA-fructose, and BSA-MGO/GO models

BSA-fructose (or MGO/GO) was prepared in phosphate buffer (PBS) (50 mM pH 7.4) containing BSA (20 mg/mL) and fructose (0.5 mol/L, or MGO/GO 10 mM). The protein solutions were incubated at 37℃ with the various concentrations of berry ethanol extracts (treatment) or without them (control). AG was used as positive control. Using 370 and 440 nm as the excitation and emission wavelengths, fluorescent AGEs were measured on a fluorescence microplate reader after 7 days (Zhao et al., 2021). Percentage of the AGE inhibition was calculated by the following equation (1):

$$\text{Inhibition of fluorescent AGEs (\%)} = [1-(F_{sample} / F_{control})] \times 100 \quad (1)$$

Measurements of antioxidant activities of berry extracts

In vitro free radical scavenging capacities of the berry samples were determined by the ABTS and peroxyl radical scavenging capacity (PSC) assays, as previously reported (Lang et al., 2021; Zang et al., 2021a). Ascorbic acid (Vit C) used as standard control, and the cellular antioxidant activity (CAA) were measured as described using HepG2 cells (Wang et al., 2017).

Fluorescence spectroscopy and synchronous fluorescence spectroscopy

The interaction of black chokeberry extracts with BSA was measured using the fluorescence quenching method at λ_{ex} = 280 nm and λ_{em} = 300–400 nm by a fluorescence spectrometer FLx800™Fluorometer. The protein solutions (0.1 mg/mL) were incubated at 37 °C with the stated quantities of black chokeberry extracts (treatment) or without them (control). In addition, the emission and excitation wavelength of the synchronous fluorescence spectrometer were set from 265 to 325 nm and 280–340 nm, respectively for Δλ15 nm. The emission and excitation wavelength were set from 280 to 420 nm and 220–360 nm, respectively for Δλ60 nm. The slit width was set at 5 nm (Lang et al., 2019).

Circular dichroism (CD) spectroscopy

The effect of black chokeberry extracts on the conformational change of glycated protein were measured by CD spectra recorded on a JASCO J-810CD spectropolarimeter (Rhode Island, USA), which scanned in the far-UV region of 190–250 nm in a quartz cuvette of 1 mm path length at 25℃ (Zang et al., 2021b).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The effect of black chokeberry extracts on protein cross-linking structure was further analyzed by SDS-PAGE according to published method (Sheng et al., 2018; Ni, Song, Pan, Gong, & Zhang, 2021).

Briefly, samples were boiled in 4 × loading buffer for 10 min and centrifuged at 10,000 g for 1 min. After loading and separating the samples onto the gel lanes, the gel was stained for 1 h with 0.05 % Coomassie blue R-250 and then for 24 h decolored with 40 % (v/v) methanol containing 10 % (v/v) acetic acid.

Quantification of the α-dicarbonyl contents

The MGO and GO content were quantified according to a published protocol with minor modifications (Zhang et al., 2021). Briefly, 1 mg/mL of derivatization reagent OPD dissolved in PBS (0.2 mol/L pH 7.4) was added to 1 mL each mode's sample at 25 ± 2℃ in dark for 12 h, followed by incubation at 37℃ for 0.5 h. The derived mixture was applied into a HSS T3 column (4.6 × 150 mm, 5 μm; Waters, USA) with the injection volume of 5 μL, the flow rate 0.3 mL/min, and the column temperature at 27℃. An aqueous solution including formic acid (0.1 %, v/v) was used as solvent A and methanol as solvent B. The gradient method was as follows: 0–2 min, 45–45 % B; 2–3.5 min, 45–80 % B; 3.5–6 min, 80–80 % B; 6–8.01 min, 80–45 % B; 8.01–10 min, 45 %-45 %. MGO and GO were then determined under the λ = 290 nm. Meanwhile, the MGO/GO residual content was quantified according to the standard curves of MGO and GO.

Determination of the MGO and GO trapping capacity

MGO/GO (10 mM) was reacted with various concentrations of black chokeberry extracts and its major compounds (cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, procyanidin B2) in PBS (0.2 mol/L pH 7.4). The derivative reaction and HPLC detecting method were mentioned in 2.7.1. MGO/GO (10 mM) was incubated with 0.8 mg/mL of black chokeberry extracts and its major compounds in PBS (0.2 mol/L pH 7.4) for 0, 0.5, 1, 2, 6, 12 and 24 h, respectively. The MGO/GO trapping percentage (%) was evaluated by the following equation (2):

$$\text{MGO/GO trapping (\%)} = [\ (\text{MGO/GO}_{\text{control}} - \text{MGO/GO}_{\text{sample}}) / \text{MGO/GO}_{\text{control}}] \times 100 \quad (2)$$

Identification of MGO or GO adducts using UPLC-Q-TOF-MS

The mixtures were filtered and subjected to LC-MS/MS analysis on an Agilent UPLC 1260-QTOF 6530 system. Chromatographic separation was performed on a Waters Cortecs C18 column (2.1 × 50 mm, 1.7 μm) with mobile phase A (water containing 10 mM acetic acid) and mobile phase B (methanol) at a flow rate of 0.3 mL/min. The injection volume was 5 μL. The gradient program of cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside and their adducts were: 0–1 min, 10 % B; 1–8 min, 10–90 % B; 8–12 min, 90 % B; 12–12.1 min, 90–10 % B; 12.1–13 min, 10 % B. Mass data were acquired in positive ion mode. The gradient program of procyanidin B2 and its adducts was: 0–5 min, 5 % B; 5–20 min, 25 % B; 20–22 min, 40–80 % B; 22–24 min, 80–100 % B. Mass data were acquired in negative ion mode. The parameters were as follows: gas temperature, 360 °C with a flow rate of 7 L/min; sheath gas temperature, 400 °C; sheath gas flow, 11 mL/min; capillary voltage, 3500 V. The mass signals were collected from 100 to 1000 *m/z* and the MS/MS spectra were obtained by "Find by Formula" analysis using Agilent MassHunter Qualitative Analysis (B06.00).

Statistical analysis

All the assays were represented by three replicates and all the date were presented as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was performed, and Duncan's test of mean comparison was assessed at the probability level of 5 % ($p < 0.05$).

Results and dicussion

Effect of berry extracts on the formation of AGEs in different reaction models

AGEs are the end products of glycation reactions, and they are produced through a series of complex reactions, including rearrangement, oxidation, dehydration, or cyclization. In this study, we evaluated the anti-glycation effect of eight berry extracts in three reaction models, namely BSA-fructose, BSA-MGO, and BSA-GO, and the results are presented in Fig. 1A-C in sequence.

The BSA-fructose model evaluates all stages of protein glycation. In the BSA-fructose model, the inhibitory effect of all tested berries on AGEs formation were shown in Fig. 1A. After incubation for 7 days, all berry extracts exhibited a significant inhibitory effect in a dose-dependent manner. Black chokeberry exhibited the highest inhibitory effect, followed by blueberry and blackcurrant. The IC_{50} values of black chokeberry, blueberry, and blackcurrant were calculated to be 0.35 ± 0.02, 0.36 ± 0.06, and 0.39 ± 0.08 mg/mL, respectively, as shown in Table 1.

During the middle stage of protein glycation, reactive carbonylation compounds such as MGO and GO are formed (Zhang et al., 2021), which can react with amino groups of proteins to form protein cross-links, critical intermediates during the generation of AGEs (Xu, You, & Zhao, 2023). The inhibitory effects of each berry extract on AGEs formation in the BSA-MGO and BSA-GO reaction models were shown in Fig. 1B-C. Similarly, the addition of different berry extracts (0.1, 0.5, 1, and 5 mg/mL) into the reaction model significantly reduced the content of AGEs in a dose-dependent manner. In the BSA-MGO model, the highest inhibitory effect was observed in blackcurrant, black chokeberry, and blueberry, with the IC_{50} values of 0.44 ± 0.06, 0.45 ± 0.03, and 0.63 ± 0.25 mg/mL, respectively. Similar results were found in the BSA-GO model. The IC_{50} values were 0.48 ± 0.11, 0.57 ± 0.12, and 0.61 ± 0.12 mg/mL, respectively, and ranked in the following order: black chokeberry, blackcurrant, and raspberry.

In summary, all eight berry extracts tested in this study showed a significant concentration dependence decrease in AGEs formation across various reaction systems. Notably, black chokeberry exhibited a novel inhibitory effect in all three reaction models, followed by blackcurrant, blueberry, and raspberry.

Chemical composition and antioxidant activity of different berry extracts

We measured the component content and antioxidant activity of selected berries, and the results are summarized in Table 1. Firstly, we analyzed the TPC, TFC, TAC, and TPAC of the eight berry extracts. The TPC and TFC of the berry extracts ranged from 80.07 ± 0.45 to 230.44 ± 1.27 mg GAE equivalents/g DW and 62.21 ± 4.63 to 132.12 ± 2.40 mg RE equivalents/g DW, respectively. Among the eight berry extracts, black chokeberry had the highest TPC value, while blueberry had the highest TFC value (132.12 ± 2.40 mg RE equivalents/g DW), followed by raspberry, sea buckthorn, and black chokeberry. These findings are consistent with previously published studies (Jurendić & Ščetar,2021; Hwang, Yoon, Lee, Cha, & Kim, 2014).

Proanthocyanidins and anthocyanins are two vital groups of compounds found in berries. The total antioxidant capacity (TAC) levels of berry extracts ranged from 9.85 ± 0.33 to 168.83 ± 1.07 mg C3G equivalents/g DW. Blueberry exhibited the highest TAC value (168.83 ± 1.07 mg C3G equivalents/g DW), followed by black chokeberry (111.58 ± 3.33 mg C3G equivalents/g DW), which is consistent with values reported in a previous study (Kim, Han, Kim, Kim, & Kim, 2021). Furthermore, our study found that black chokeberry had the highest TPAC among all the tested berries, with a content of 42.54 ± 6.94 mg PCB2/g DW, almost four times that of blueberry. Cranberry had the second-highest TPAC content at 33.13 ± 2.86 mg PCB2/g DW, while the content of other berries ranged from 7.87 ± 2.93 to 14.17 ± 3.76 mg

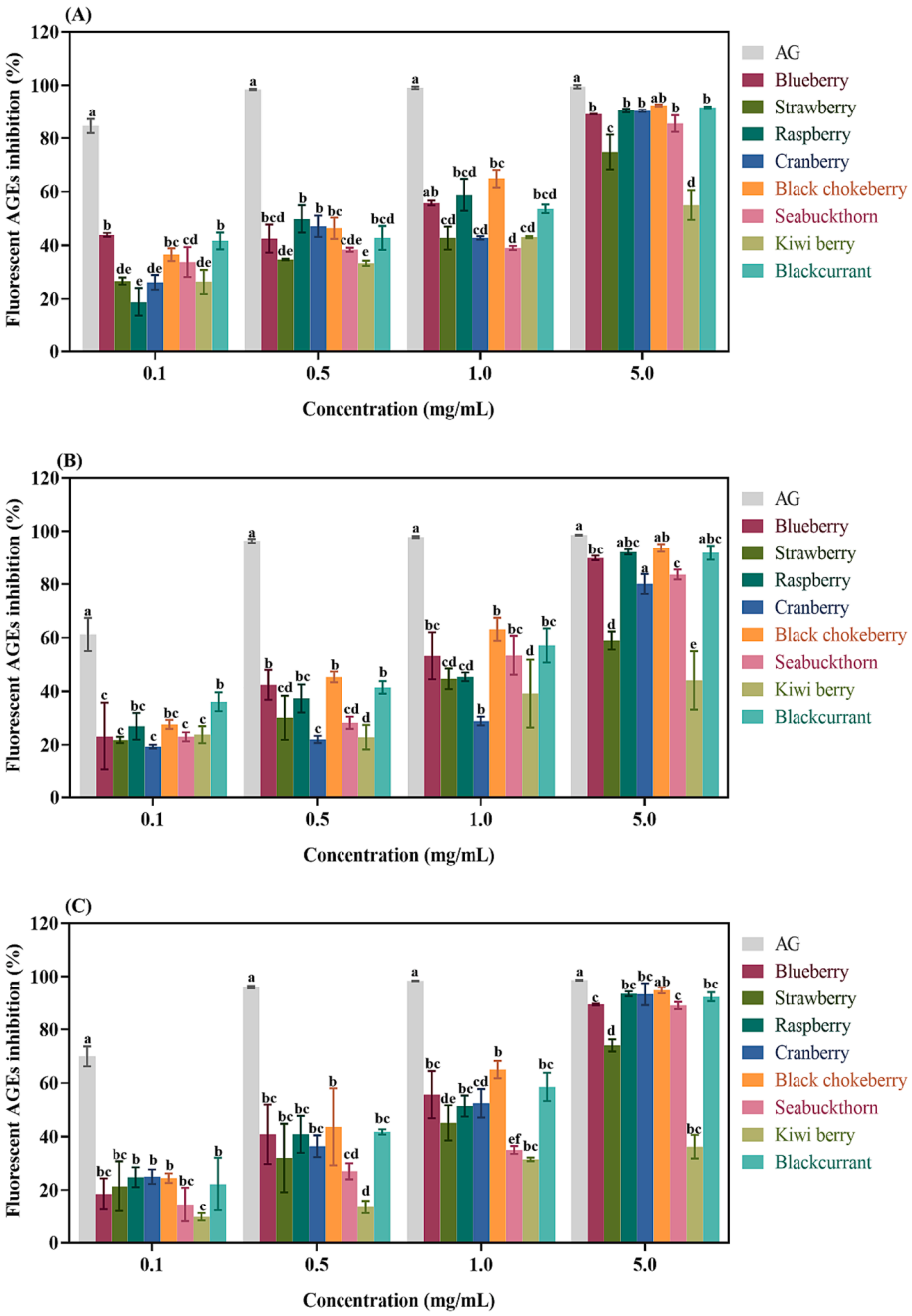


Fig. 1. Inhibition rate of AG and berry extracts on fluorescent AGEs in different models. (A) BSA-fructose model, (**B**) BSA-MGO model, (**C**) BSA-GO model.

PCB2/g DW (Taheri, 2013; Wangensteen et al., 2014).

We also analyzed the cellular and *in vitro* radical scavenging activity of the eight berry extracts using the ABTS, PSC, and CAA methods. The results showed that the IC_{50} values of ABTS free radical scavenging capacity of the berries ranged from 0.13 ± 0.01 to 1.13 ± 0.02 mg/mL. Black chokeberry exhibited the most significant ABTS antioxidant capacity, followed by blueberry, as expected. The PSC values of the berries ranged from 0.12 ± 0.05 to 0.64 ± 0.08 μmol Vc equiv./mg. Blackcurrant had the highest PSC value (0.64 ± 0.08 μmol Vc equiv./mg), followed by blueberry (0.40 ± 0.09 μmol Vc equiv./mg) and black chokeberry (0.33 ± 0.08 μmol Vc equiv./ mg). In the cellular antioxidant assay, blueberry and black chokeberry also showed high CAA values. Therefore, we speculated that the chemical composition content and antioxidant activity of berries may be one of the reasons for their anti-glycation activity. However, the association between the antioxidant properties of the investigated berry extracts and their anti-glycation properties varies when taking into account the inhibitory effect of berries on AGEs in various simulated glycation reaction systems.

Effect of black chokeberry extracts on the fluorescence spectra of BSA

Based on the above analysis, black chokeberry demonstrated the highest anti-glycation activity throughout all stages of the glycation process, and the exact anti-glycation mechanism has yet to be fully interpreted. To further investigate this mechanism, we utilized the intrinsic fluorescence spectrum to study the binding behaviors between polyphenols from black chokeberry extract and BSA. The resulting data is presented in Fig. 2, where it can be observed that the fluorescence intensity of BSA at 340 nm decreased gradually as the concentration of black chokeberry increased, indicating a dose-dependently fluorescence quenching (Fig. 2A).

Fluorescence quenching can be categorized into two types: dynamic

Table 1
Anti-glycation, phenolic composition, and antioxidant capacities of different berry extracts.

Sample	Total AGEs*			Chemical composition				Antioxidant activity		
	BSA-Fructose	BSA-MGO	BSA-GO	TPC (mg GAE/g DW)	TFC (mg RE/g DW)	TAC (mg C3G/g DW)	TPAC (mg PCB2/g DW)	ABTS*	PSC (μmol Vc equiv./mg DW)	CAA (μmol QE/100 g DW)
Blueberry	0.36 ± 0.06^{b}	0.63 ± 0.25^{b}	0.68 ± 0.22^{b}	200.39 ± 0.26^{c}	132.12 ± 2.40^{a}	168.83 ± 1.07^{a}	4.20 ± 1.18 ^{h}	0.23 ± 0.07^{d}	0.40 ± 0.09^{b}	117.56 ± 5.44^{a}
Strawberry	1.12 ± 0.13^{b}	2.29 ± 0.87^{b}	1.20 ± 0.46^{b}	110.79 ± 0.09 ^{g}	62.21 ± 4.63 ^{h}	50.41 ± 0.53^{d}	7.87 ± 2.93 ^{g}	0.39 ± 0.08^{bc}	0.21 ± 0.09^{de}	38.31 ± 37.59^{c}
Raspberry	0.54 ± 0.06^{b}	0.69 ± 0.13^{b}	0.61 ± 0.12^{b}	220.11 ± 0.47^{b}	124.07 ± 4.40^{b}	9.85 ± 0.33^{f}	12.34 ± 1.01^{d}	0.38 ± 0.02^{bc}	0.12 ± 0.05^{e}	30.99 ± 30.15 ^{cd}
Cranberry	0.63 ± 0.07^{b}	1.64 ± 0.20^{b}	0.64 ± 0.05^{b}	180.56 ± 0.36^{f}	110.08 ± 4.43^{f}	28.21 ± 3.79 ^{e}	33.13 ± 2.86^{b}	0.36 ± 0.02^{c}	0.22 ± 0.07^{de}	–
Black chokeberry	0.35 ± 0.02^{b}	0.45 ± 0.03^{b}	0.48 ± 0.11^{b}	230.44 ± 1.27^{a}	113.22 ± 4.95^{d}	111.58 ± 3.33^{b}	42.54 ± 6.94^{a}	0.13 ± 0.01^{e}	0.33 ± 0.08^{bc}	88.59 ± 2.10^{ab}
Sea buckthorn	0.76 ± 0.15^{b}	0.86 ± 0.10^{b}	1.16 ± 0.07^{b}	190.88 ± 0.31^{d}	120.68 ± 5.43^{c}	–	11.17 ± 8.24^{e}	1.13 ± 0.02^{a}	0.13 ± 0.01^{e}	91.77 ± 4.41^{ab}
Kiwi berry	2.92 ± 1.00^{b}	–	–	80.07 ± 0.45 ^{h}	84.05 ± 30.21 ^{g}	–	10.49 ± 1.54^{f}	0.45 ± 0.03^{b}	0.28 ± 0.08^{bc}	–
Blackcurrant	0.39 ± 0.08^{b}	0.44 ± 0.06^{b}	0.57 ± 0.12^{b}	190.22 ± 0.35^{e}	110.74 ± 2.84^{e}	103.4 ± 0.25^{c}	14.17 ± 3.76^{c}	0.46 ± 0.04^{b}	0.64 ± 0.08^{a}	55.54 ± 16.49^{bc}
AG	0.026 ± 0.02^{a}	0.069 ± 0.01^{a}	0.048 ± 0.01^{a}	–	–	–	–	–	–	–

*Represents IC_{50} value with the unit (mg/mL); GAE, RE, C3G, PCB2, Vc and QE represents gallic acid equivalent, rutin equivalent, Cyanidin-3-*O*-glucoside, procyanidin B2, vitamin C and quercetin equivalent, respectively. Different letters suggested significant difference within the same column s($p < 0.05$, Duncan's test.

and static quenching. To understand the potential quenching mechanism, the data were calculated using the Stern Volmer Plot as shown in Fig. 2B-D. The K_q value was determined to be 1.96 ± 0.20 × 10^{11} $L{\cdot}mol^{-1}{\cdot}s^{-1}$, which is significantly higher than the maximum diffusion-limited quenching constant (2 × 10^{10} $L{\cdot}mol^{-1}{\cdot}s^{-1}$), indicating that the quenching process of BSA with black chokeberry polyphenol occurred through a static quenching mechanism. Additionally, the double-logarithmic regression plot was employed to calculate the binding constant Ka and binding stoichiometry n. The binding affinity constant K_a was found to be 1.24 ± 0.01 (×10^5 $L{\cdot}mol^{-1}$), suggesting a strong binding effect between black chokeberry extracts and BSA. Furthermore, the double logarithmic regression plot of BSA exhibited a well-fined linear relationship with a binding site value of approximately 1. This suggests that BSA has one class of binding sites for black chokeberry polyphenol compounds.

Synchronous fluorescence spectrum is a technique derived from fluorescence spectrum and is commonly used to study protein conformation changes, providing insights into the polarity of the microenvironment surrounding amino acid residues. When Δλ equals 15 nm and 60 nm, it reflects the microenvironment around tyrosine and tryptophan residues, respectively (Lang et al., 2019). As shown in Fig. 2E-F, the fluorescence intensity of tyrosine and tryptophan gradually decreases with the increasing concentrations of black chokeberry extracts. Notably, the fluorescence intensity of tryptophan residue was higher than that of tyrosine residue, indicating that tryptophan served as the main fluorescence source of BSA. Both amino acids exhibited a slight red shift in the maximum emission wavelength, indicating a decrease in hydrophobicity and an increase in the polarity of tyrosine and tryptophan residues. These findings suggest that black chokeberry can interact with BSA, indirectly reducing the formation of AGEs.

Effect of black chokeberry extracts on protein cross-linking and secondary structure

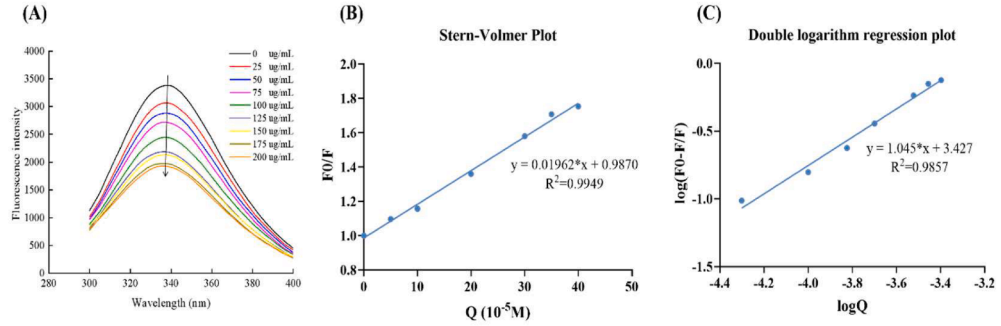
SDS − PAGE was used to analyze the effects of glycation and black chokeberry extracts on the molecular weight (MW) of BSA. In Fig. 2G, native BSA exhibited a distinct band at 66.7 kDa. However, the MW of glycosylated BSA altered, and a high molecular weight band appeared at 97.4 kDa, indicating that fructose-induced non-enzymatic glycosylation of protein formed a high molecular cross-linked structure of a protein. Interestingly, with the addition of black chokeberry extracts, the band gradually became clearer as the extract concentration increased, which was also confirmed by the densitometric analysis of the bands (Fig. 2H). These results suggest that the black chokeberry extracts may have played a role in preventing protein cross-linking and aggregate formation, thereby inhibiting the glycosylation reaction and reducing the generation of AGEs.

CD spectra were used to determine the impacts of glycation on the secondary structure of BSA. As shown in Fig. 2I, native BSA spectra exhibited two distinct negative peaks at 208 nm and 222 nm. However, the negative absorption band of glycated BSA decreased, accompanied by a shift in position and a reduction in signal strength. Fig. 2J demonstrates that the treatment of black chokeberry extracts alleviated the signal intensity of glycated BSA. The contents of α- helix and β- sheet in the native BSA was 36.1 % and 16.5 % respectively. Following glycation, the α-helix content decreased to 33.8 %, while the β-sheet content increased to 18.8 %, indicating a structural transformation from α-helix to β-sheet caused by the glycation process. After treatment with black chokeberry, the α-helix and β-Sheet contents of glycated BSA were recovered to 35.9 % and 18.2 %, respectively.

Capture ability of black chokeberry extract on MGO/GO

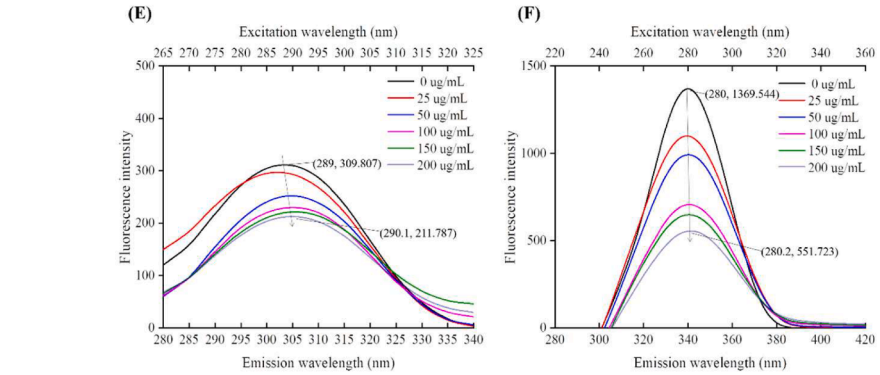
MGO and GO are carbonyl compounds that serve as intermediate products of glycation reaction and important precursors in promoting the formation of AGEs. Therefore, we investigated the effect of different concentrations of black chokeberry extract on the elimination efficiency of MGP and GO after a 24 h incubation under simulated conditions. As shown in Fig. 3A, after treatment with different concentrations of black chokeberry extracts, the residual levels of MGO or GO in models were significantly lower than the control in a dose-dependent manner. At a concentration of 0.1 mg/mL of black chokeberry extract, the residual content of GO (0.46 ± 0.02 mg/mL) was approximately four times higher than that of MGO (0.12 ± 0.01 mg/mL), indicating that the inhibition of MGO was more sensitive compared to GO. These results suggest that black chokeberry extract may effectively mitigate AGEs formation by trapping the α-dicarbonyl compounds, particularly MGO.

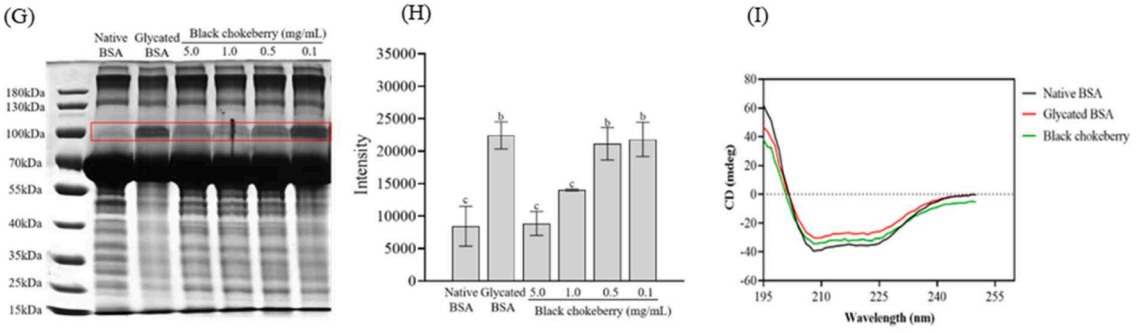
The trapping ability of black chokeberry extract on MGO and GO was presented in Fig. 3B-C to further demonstrate whether the reduction is attributable to the direct trapping effect of black chokeberry extract on MGO and GO. As we expected, black chokeberry extracts trapped MGO and GO in a dose- and time- dependent manner. At a concentration of 0.8 mg/mL of black chokeberry extract, the trapping rate of MGO and GO was 41.95 % and 55.32 %, respectively. As shown in Fig. 3D. At 24 h,



(D)

T (K)	K_{sv} ($\times10^3$ L·mol^{-1})	K_q ($\times10^{11}$ L·mol^{-1}·s^{-1})	R^2	K_a ($\times10^5$ L·mol^{-1})	n	R^2
310	1.96±0.20	1.96±0.20	0.9949	1.24±0.01	1.05	0.9857





(J)

Secondary structure	Native BSA	Glycated BSA	Black chokeberry
α-Helix	36.1%	33.8%	35.9%
β-Sheet	16.5%	18.8%	18.2%

Fig. 2. (**A**) Fluorescence spectra of BSA in the presence of 0–200 μg/mL black chokeberry at pH 7.4, 310 K. (**B**) The Stern-Volmer plots for the quenching of BSA by black chokeberry. (**C**) Double logarithmic regression of BSA with black chokeberry at different concentrations. (**D**) Quenching and binding constants for the interactions between BSA and black chokeberry at different concentrations. (**E-F**) Synchronous fluorescence spectra of BSA with the addition of black chokeberry extracts at 298 K when (E) $\Delta\lambda = 15$ nm and (F) $\Delta\lambda = 60$ nm. (**G**) SDS-PAGE profiles of BSA with black chokeberry extracts. (H)The densitometric analysis of glycation BSA with black chokeberry. (**I**) CD spectra of BSA and the Glycated BSA with and without black chokeberry extracts. (**J**)Effects of black chokeberry on the secondary structure contents of glycated BSA.

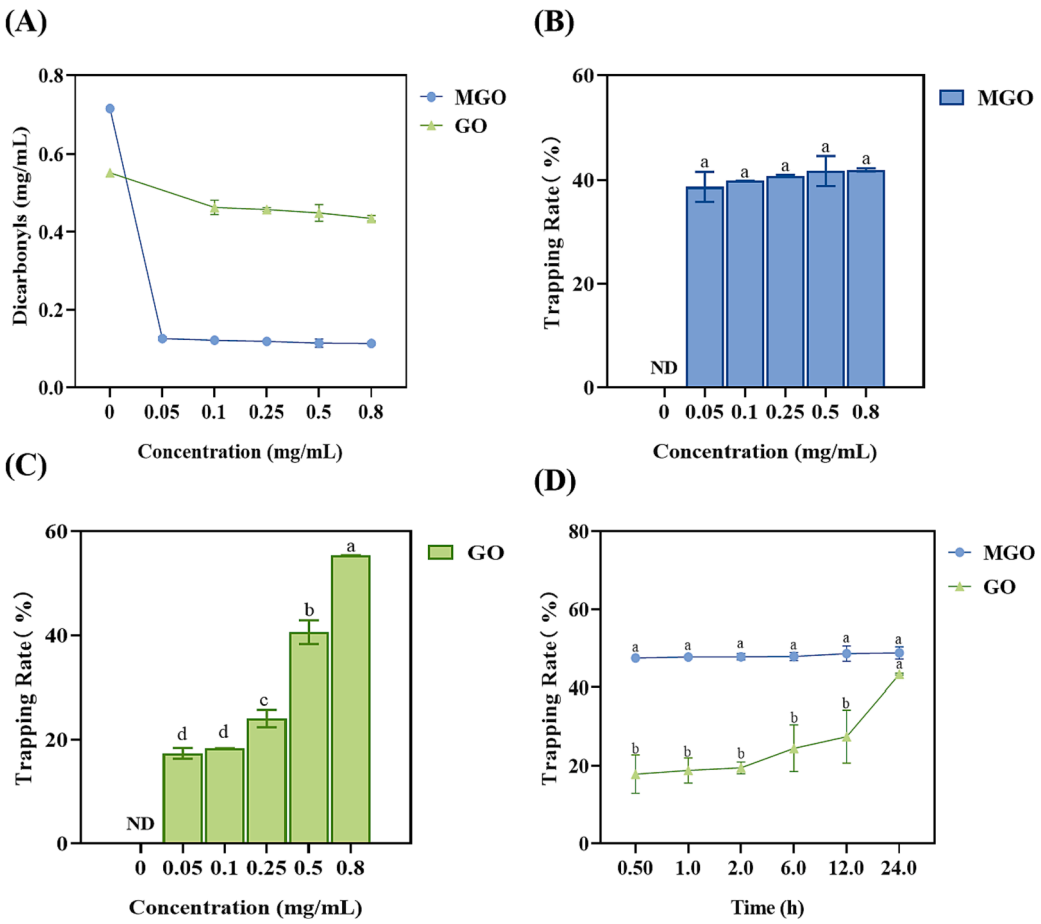


Fig. 3. (**A**) Effects of black chokeberry on the content of MGO and GO in the BSA-MGO/GO models; (**B-C**) Trapping of MGO and GO by black chokeberry extract at different concentrations; (**D**) Trapping of MGO and GO by black chokeberry extract at different times.

with a black chokeberry extract concentration of 0.8 mg/mL, the trapping efficiency for MGO reached 48.80 %, while that of GO was 43.34 %. The results suggest that black chokeberry extract efficiently traps MGO. One potential reason for this is that glyoxal mainly exists in aqueous solutions in the form of a dimer or trimer, which slows down its conversion to free GO, and thus decelerates the trapping reaction between black chokeberry extract and GO. Similar findings have been reported in a previous study (Li, Zheng, Sang, & Lv, 2014). These findings suggest that reactive carbonyl compounds can be effectively captured by black chokeberry extract.

Trapping capacity of major compounds from black chokeberry extract on MGO/GO and identification analysis of adducts

In our previous research (Zang et al., 2021b), we found that the dominant polyphenol compounds in black chokeberry were cyanidin-3-*O*-arabinoside, cyanidin-3-*O*-galactoside, and procyanidin B2, in that order. We measured the capture capacity of these three major compounds for MGO/GO (as shown in Fig. 4A-C), and they trapped MGO and GO in a dose-dependent manner. At a concentration of 75 μg/mL, the trapping rate of MGO by cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, and procyanidin B2 was 56.85 %, 56.95 %, and 68.27 %, respectively. The trapping rate of GO was 63.60 %, 54.55 %, and 50.16 %, respectively. The dynamic trapping process of carbonyl compounds showed an increase in trapping capacity with incubation time. At 24 h of incubation time, the trapping rate of MGO by cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, and procyanidin B2 was 56.54 %, 42.73 %, and 54.09 %, respectively. Meanwhile, the trapping rate of GO was 23.70 %, 21.87 %, and 55.13 %, respectively. These results indicate that all three compounds were more effective in trapping MGO than GO.

The high MGO/GO trapping efficiency may be attributed to the formation of adducts between the major compounds found in black chokeberry and α-dicarbonyls. After the complete reaction of the three compounds (cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, and procyanidin B2) with MGO/GO, the secondary fragment ions and possible structure of the reaction adducts have been further characterized and identified using UPLC-Q-TOF-MS and the results were shown in F ig. 6. It is known that the major fragment ions of cyanidin-3-*O*-galactoside (*m/z* 449 $[M + H]^+$) are *m/z* 287 $[M + H\text{-Gal}]^+$, resulting from the loss of sugar moiety. When cyanidin-3-*O*-galactoside was incubated with MGO, the resulting molecular ions had a mass of *m/z* 593 $[M + H]^+$, corresponding to cyanidin-3-*O*-galactoside-diMGO (mass shift + 144 *m/z*). The MS^2 spectra showed a product ion at *m/z* 521(mass shift −72 *m/z*) due to the loss of the MGO molecule. The ion at *m/z* 431 $[M + H\text{-Gal}]^+$ and 395 $[M + H\text{-Gal-18–18}]^+$ was attributed to the di-MGO-cyanidin aglycone and the loss of two water molecules, respectively. Similarly, following incubation with GO, cyanidin-3-*O*-galactoside-diGO was identified with a similar fragmentation pattern (565 $[M + H]^+ \rightarrow m/z$ 507(mass shift −58 *m/z*) → 345 $[M + H\text{-Gal}]^+$) (Fig. 5A).

In comparison, cyanidin-3-*O*-arabinoside (419 $[M + H]^+$) reacted with MGO or GO to generate mono adducts. The MS^2 spectra for mono adducts exhibited cyanidin-3-*O*-arabinoside-monoMGO (491 $[M + H]^+$) and cyanidin-3-*O*-arabinoside-monoGO (477 $[M + H]^+$). The loss of sugar moiety (-*m/z* 132) resulted in the cyanidin-3-*O*-arabinoside-monoGO fragment ion at *m/z* 345 $[M + H\text{-Ara}]^+$ (Fig. 5B). Previous studies suggest that the major trapping sites of α-dicarbonyl compounds are the C6 and C8 positions on the A-ring (Li, Zheng, Sang, & Lv, 2014; Zhang et al., 2022). Furthermore, prior research has demonstrated that

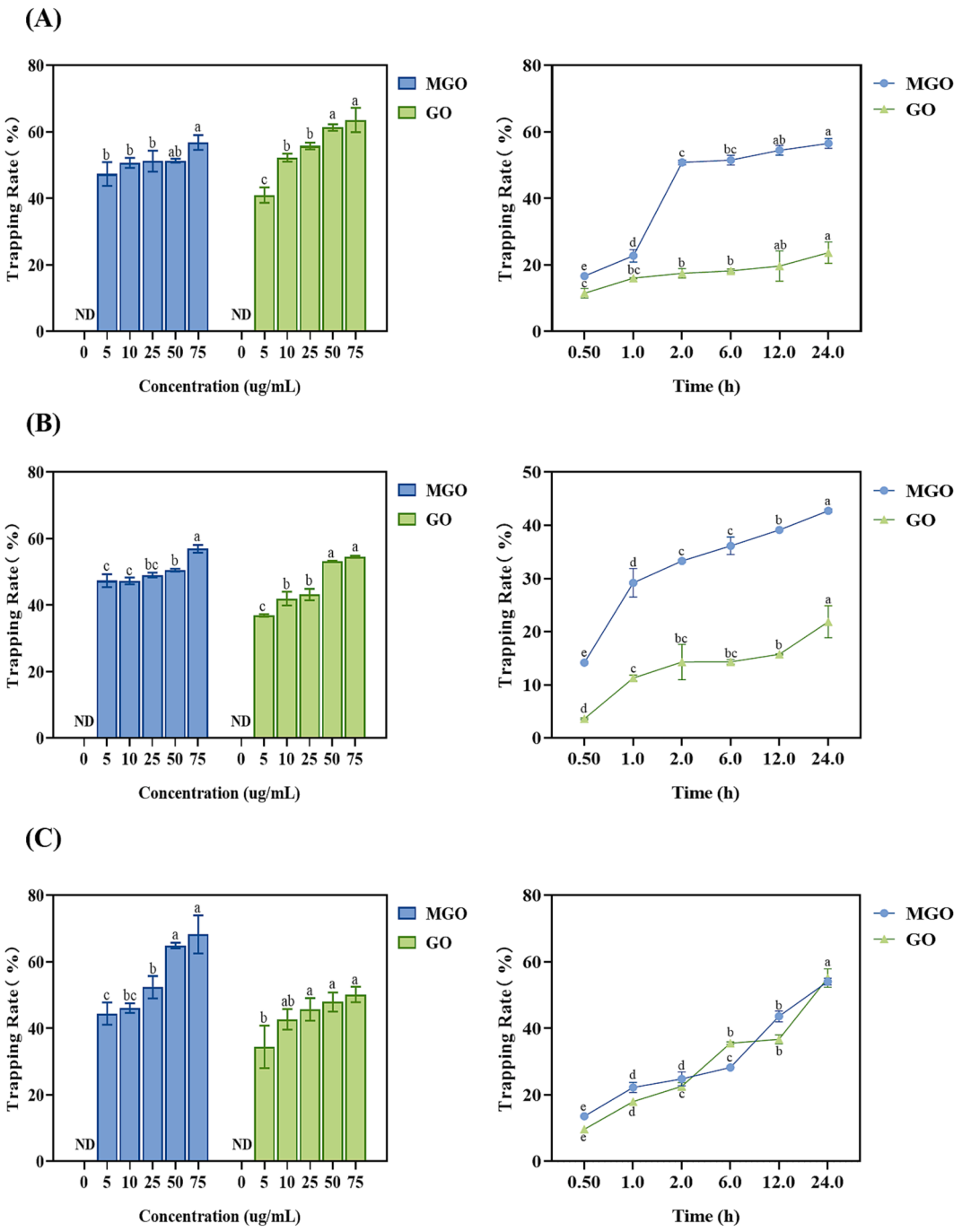


Fig. 4. (**A**) Trapping capacity of MGO and GO by cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside and procyanidin B2 (**A-C**) at different concentrations and at different times at 37°C.

quercetin can rapidly trap MGO and inhibit the formation of AGEs by forming mono- and di-MGO adducts, with the di-MGO adducts becoming the dominant products during prolonged incubation (Li, Zheng, Sang, & Lv, 2014).The intensity of the di-MGO-myricetin adduct peaks increases, while the intensity of the mono-MGO-myricetin adduct peaks decreases as the amount of MGO increases in the reaction (Zhang, Xiao, Lv & Sang, 2020). Overall, we speculate that the quantity of mono- and di-MGO/GO adducts are related to reaction time and reaction ratio. Further kinetics studies of each compound in the trapping reaction with MGO and GO are needed.

Procyanidin B2 is known to have a molecular weight of 578. Based on the *m/z* molecular ion peak data, it is speculated that there are three types of adduct products when procyanidin B2 reacts with MGO: procyanidin B2-monoMGO (*m/z* 649 $[M-H]^-$), procyanidin B2-diMGO (*m/z* 721 $[M-H]^-$), and procyanidin B2-triMGO (*m/z* 793 $[M-H]^-$). Similarly, following incubation with GO, three adduct products were also observed: procyanidin B2-monoGO (*m/z* 635 $[M-H]^-$), procyanidin B2-diGO (*m/z* 693 $[M-H]^-$), and procyanidin B2-triGO (*m/z* 751 $[M-H]^-$). However, due to the complex composition of procyanidin B2, we have provided only the molecular ion peak of each adduct. In future studies, it is advisable to consider using specific procyanidins with more defined structures to accurately identify the formed adducts.

Conclusion

In this study, we investigated the anti-glycation effect of eight kinds

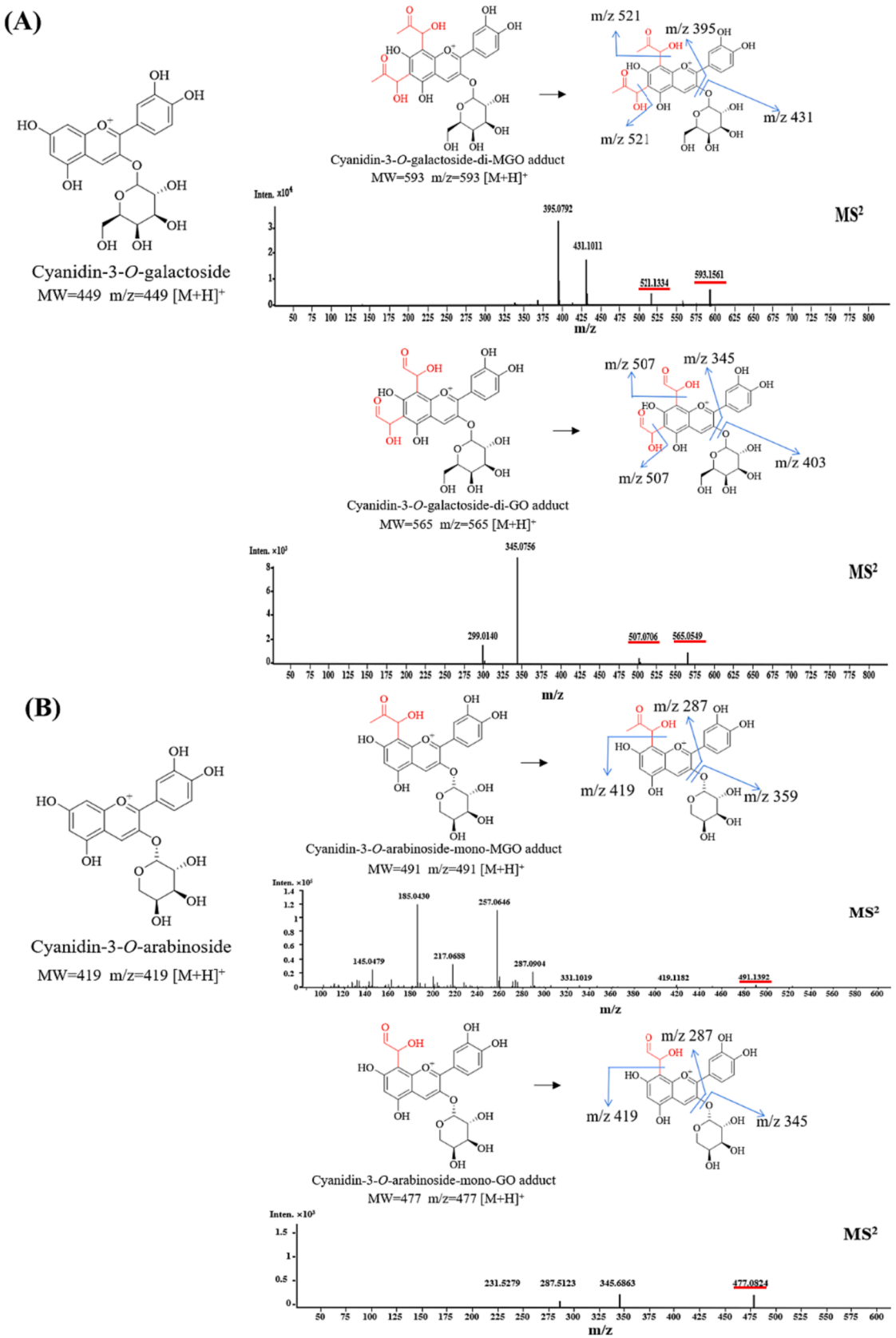


Fig. 5. The MS/MS spectra and proposed structures of tentatively identified adducts (**A**) Cyanidin-3-*O*-galactoside-di-MGO/GO adducts (**B**) Cyanidin-3-*O*-arabinoside-mono-MGO/GO adducts (**C**) Procyanidin B2-mono, di-and tri-MGO/GO adducts.

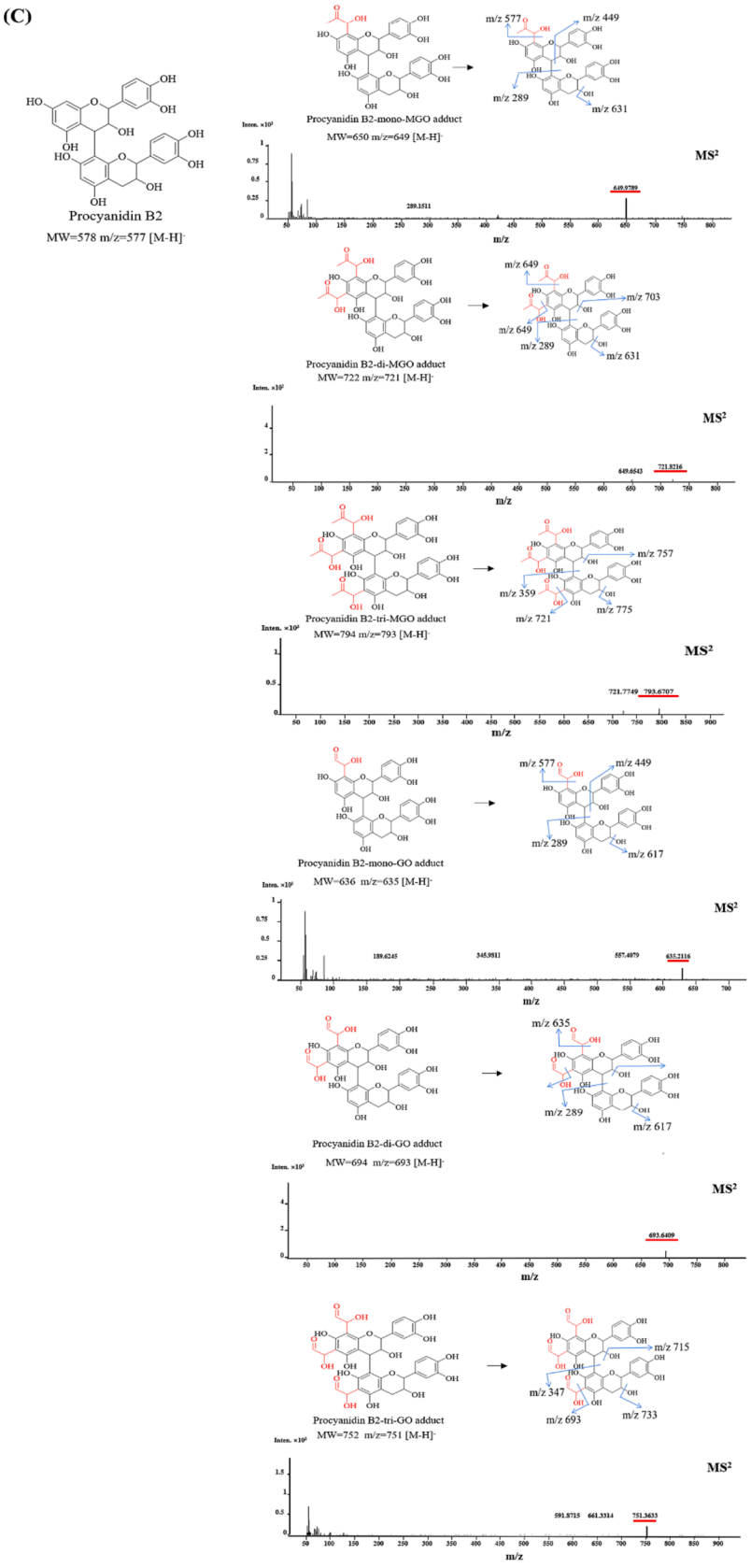


Fig. 5. (*continued*).

of berries at all stages. Among them, black chokeberry demonstrated a novel inhibitory activity against AGEs formation. The underlying mechanism behind the inhibitory effect of black chokeberry may be attributed to its binding interaction with protein, thereby preventing structural changes and inhibiting protein cross-linking. Moreover, black chokeberry, along with its three major polyphenols, namely cyanidin-3-

O-galactoside, cyanidin-3-*O*-arabinoside, and procyanidin B2, exhibited remarkable trapping capabilities for MGO and GO contents, which are the important inhibition mechanisms for AGEs formation. Through UPLC-Q-TOF-MS analysis, we were able to detect mono- or di- MGO/GO adduct of cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside, as well as procyanidin B2-mono-, di-, and tri-MGO/GO adducts. In summary, our findings clearly demonstrate that berries can serve as a promising resource for identifying novel compounds that inhibit AGEs formation. Black chokeberry, in particular, stands out as a potent anti-glycation berry, black chokeberry can be utilized in food processing to develop products with inherent properties against glycation and aging, such as beverages, gummies, and fruit powders. Moreover, It can also be used in the medical field to prevent and treat age-related chronic diseases, which has a wide prospect of applications.

CRediT authorship contribution statement

Hui Tan: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. **Baoyue Cui:** Conceptualization, Data curation, Methodology, Validation, Writing – original draft. **Kexin Zheng:** Methodology, Software. **Ningxuan Gao:** Methodology, Software. **Xuening An:** Validation. **Yu Zhang:** Validation. **Zhen Cheng:** Validation. **Yujie Nie:** Validation. **Jinyan Zhu:** Validation. **Li Wang:** Validation. **Kuniyoshi Shimizu:** Writing – review & editing. **Xiyun Sun:** Writing – review & editing. **Bin Li:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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