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Original Article

The inhibition of advanced glycation end-products by five fractions and three main flavonoids from *Camellia nitidissima* Chi flowers

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ABSTRACT

Camellia nitidissima Chi (CNC), belonging to *Camellia* genus (Theaceae family), is a medicinal and edible plant in China. Among the whole plant, the CNC flowers are especially precious, but the biological activities and the compositions of the CNC flowers are unknown. In this study, inhibiting effects on the formation of advanced glycation end-products (AGEs) of five CNC flowers fractions and three isolated compounds were investigated, these three compounds are two flavonoid glycosides and one flavanol, namely kaempferol 3-O-[2,3,4-Tri-O-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-O-acetyl-α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside, kaempferol 3-O-[2,3,4-Tri-O-acetyl-α-L-rhamnopyranosyl-(1→3)-4-O-acetyl-α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside and catechin. Among these five fractions, the ethyl acetate fraction showed the highest total phenolic contents and inhibiting effects on AGE formation. Bovine serum albumin (BSA)-glucose and BSA-methylglyoxal assay showed that the ethyl acetate fraction inhibited AGE formation by 74.49% and 34.3% at 1 mg/mL, respectively. As the main components, these three compounds also showed remarkable inhibiting effects on AGE formation by scavenging methylglyoxal, next two catechin-carbonyl adducts were identified using HPLC-ESI-MS/MS. The results showed that the CNC flowers had remarkable inhibiting effects on the formation of AGEs. The primary structure-activity relationship showed (1) the glycosides could reduce the inhibiting effects compared to kaempferol and (2) the acetyl at position 2‴ in compound **1** had no remarkable influence of the inhibiting effects on AGE formation compared to compound **2**.

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1. Introduction

Advanced glycation end-products (AGEs) are the nonenzymatic reaction final products between reducing sugars and amino groups in proteins, lipids, and nucleic acids [1]. AGEs and reactive carbonyl species play an important role in many human diseases such as diabetic complications [1], Alzheimer's disease [2], aging [3] and atherosclerosis [4]. Thus, it is a potential therapeutic method for the diabetic prevention or other pathogenic complications to search AGE inhibitors. Comparing with synthetic compounds, natural products have been proven relatively safe for human. Hence, some plant extracts have been evaluated for their inhibiting effects on the formation of AGEs in recent years [5–9]. Most of these plant extracts with the inhibiting effects on the AGE formation mainly contained the large amount of phenolic compounds in their phytochemicals [10–12]. As a major kind of phenolic compounds, flavonoid and flavonoid glycosides have been isolated from many plants, especially from some kinds of teas [13–15]. And the flavonoid and flavonoid glycosides show many biological activities in *vivo* and in *vitro*, especially flavonoid and flavonoid glycosides are regarded as AGEs inhibitors to represent a potential therapeutic target to prevent and treat diabetic complications [10,16,17].

Yellow *Camellia* includes over 42 species and 5 variants and they are mainly distributed in a narrow region of Guangxi province in Southern China and Northern Vietnam. The yellow petals of the flowers are rarely found in the world, so it is called "the pandas in plant kingdom" [14]. *Camellia nitidissima* Chi (CNC) belongs to *Camellia* genus and is regarded as a medicinal and edible plant in China. CNC plays an important role in human health [18], and the CNC leaves have the ability of inhibiting the formation of AGEs [8]. Since the CNC flowers are more rare and precious than the leaves, most of the studies about CNC are on the leaves, and the biological activities and the compositions of the CNC flowers are unknown. In this study, we isolate the major flavonoid glycosides and flavanol from the CNC flowers fractions, and evaluate the inhibiting effects on the AGE formation of CNC flowers fractions and the major flavonoid glycosides and flavanol.

2. Materials and Methods

2.1. Instruments and reagents

The nuclear magnetic resonance data (^{1}H-NMR and ^{13}C-NMR) were recorded on Bruker AV-500 (Bruker Inc., Germany). The mass spectrometry (MS) spectra were performed on Agilent 1100 Series LC-MSD-Trap/SL and Thermo TSQ Quantum LC/MS spectrometers. Silica gel (100–200 mesh, 200–300 mesh), which was used for silica gel column chromatography and thin-layer chromatography was purchased from Qingdao Marine Chemical Factory (Qingdao, China). Sephadex LH-20 (GE Healthcare Bio-sciences AB, Uppsala, Sweden), C18 (YMC, Japan) and RP-18 F254 plates (0.25 mm, Merck, Germany) were used. Methylglyoxal was purchased from Wuhan Huameihua Co. (Wuhan, China). Aminoguanidine was purchased from Dulai Biotechnology Co. (Nanjing, China). Bovine serum albumin (BSA) was purchased from Beijing Solarbio Science and Technology Co. (Beijing, China). HPLC grade methanol was purchased from Tedia (Fairfield, USA). All other chemicals were analytical grade and purchased from Shuangling Chemical Reagent Co. (Nanjing, China).

2.2. Plant materials

The *C. nitidissima* Chi (CNC) flowers were collected in July 2013 from Fangchenggang, Guangxi Province, China. The flowers were air-dried and coarsely powdered (ca. 40 mesh).

2.3. Determination of total phenolic contents

The total phenolic contents were determined by the Folin–Ciocalteu method following the literature [19].

2.4. The isolation of phytochemicals

The CNC flowers (6 kg) were refluxed with 95% ethanol for 3 times (3, 2, and 1 h, respectively). Ethanolic extract (1200 g) was obtained through a rotary evaporator at 45 °C. Then the ethanolic extract was suspended in water and partitioned by dichloromethane (3 × 4.5 L), ethyl acetate (3 × 4.5 L) and n-butanol (3 × 4.5 L), respectively, to yield the dichloromethane (52 g), ethyl acetate (256 g) and n-butanol (560 g) fractions. The water phase was dried at 50 °C to yield the water fraction (300 g). The dichloromethane fraction was subjected to silica gel column chromatography eluted with dichloromethane-methanol (1:0, 49:1, 25:1, 15:1, 9:1, 5:1, 0:1) gradient system to yield 4 subfractions on the basis of thin-layer chromatography analysis. The subfraction 3 was subjected to silica gel column chromatography, Sephadex LH-20 (dichloromethane-methanol 1:1) and C18 (methanol-water 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 10:0) repeatedly, to yield compound **1** (3.25 g). Then the compound **2** (2.16 g) and compound **3** (2.57 g) were obtained from the ethyl acetate fraction through the similar methods.

2.5. Antiglycation assay in BSA-glucose model

The antiglycation assay in BSA-glucose model was tested using the published method [8] with minor modifications. The final concentrations of catechin were 5 mg/mL, 2.5 mg/mL, 1 mg/mL and 0.2 mg/mL. Fluorescence intensity (excitation wavelength was monitored at 340 nm and emission wavelength was monitored at 420 nm) of the test solution was measured on a microplate reader (TECAN Infinite 200 Pro., Austria).

2.6. Antiglycation assay in BSA-methylglyoxal model

The antiglycation assay in BSA-methylglyoxal model was carried out based on the published method [8] with minor modifications. The final concentrations of catechin were 5 mg/mL, 2.5 mg/mL, 1 mg/mL and 0.2 mg/mL. Fluorescence intensity (excitation wavelength was monitored at 340 nm and emission wavelength was monitored at 420 nm) of the test solution was measured on a microplate reader (TECAN Infinite 200 Pro., Austria).

2.7. Methylglyoxal scavenging assay

The methylglyoxal trapping assay followed a published method with some modifications [5,8]. Methylglyoxal (10 mM), o-phenylenediamine (50 mM) were freshly prepared in phosphate buffer (50 mM, pH 7.4). The final concentration of test samples (the five fractions, compounds **1**–**3** and kaempferol) and aminoguanidine was 5 mg/mL. Three mL methylglyoxal were incubated with 0.75 mL test samples or phosphate buffer in pH 7.4 at 37 °C water bath for 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h. After incubation, 200 μL of o-phenylenediamine were added with 200 μL of each test solution. Before HPLC analysis, the mixtures were kept in dark at room temperature for 1 h. The remaining methylglyoxal was detected on an Thermo Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc., Massachusetts, USA) equipped with a diode array detector, and a Thermo Syncronis-C18 column (250 mm × 4.6 mm i.d., 5 μm; Thermo Fisher Scientific Inc., Massachusetts, USA). Mobile phases were composed of 0.1% formic acid in water (A) and methanol (B). The elution started at 50% B and then increased to 55% in 10 min, and then increased to 100% in 11 min and lasted for 5 min, then returned to 50% in 16.5 min and lasted for 5 min. The flow rate was 1 mL/min and the injection volume was 20 μL. Detection wavelength was monitored at 315 nm.

2.8. Identification of catechin-carbonyl adducts on HPLC-ESI-MS/MS

The catechin-carbonyl adducts were identified using a published method [8] with some modifications. Catechin and methylglyoxal were dissolved in dimethyl sulfoxide and phosphate buffer (pH 7.4) to the final concentrations at 5 mg/mL, respectively. Then, methylglyoxal and catechin were mixed with equal volume and incubated at 37 °C water bath for 4 h. The instruments here were the same as the literature [8]. But mobile phase A was 0.1% formic acid of water and mobile phase B was 0.1% formic acid of methanol. The flow rate was 0.4 mL/min and the injection volume was 20 μL. The linear gradient was: 0–2 min, 5–5% B; 2–15 min, 5–30% B; 15–20 min, 30–90% B; 20–25 min, 90–90% B; 25–25.1 min, 90–5% B; 25.1–30 min, 5–5% B. Detection wavelength was set at 280 nm.

2.9. Statistical analyses

All experiments were conducted independently in triplicate, and experimental results were expressed as mean ± standard deviation or average. One-way analysis of variance (ANOVA) and Duncan's multiple range test were performed by SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) software. Statistical significance was determined by $p < 0.05$.

3. Results and Discussion

3.1. Total phenolic contents in the fractions of CNC flowers

Total phenolic contents (Table 1) of the ethyl acetate fraction was highest and that of the water fraction was the lowest.

Table 1 – The total phenolic contents of the five fractions of CNC flowers.

Fraction	Total phenolic contents (mg GAE/g)
Ethanolic extract	170.74 ± 1.984b
Dichloromethane fraction	85.02 ± 0.873c
Ethyl acetate fraction	345.14 ± 4.048a
n-butanol fraction	164.19 ± 3.175b
Water fraction	31.69 ± 1.746d

Each value was expressed as mean ± SD (n = 3). Means with different small letters (a, b, c, d) within a column were significantly different ($p < 0.05$).

Total phenolic contents of the n-butanol fraction were similar to that of the ethanolic extract. Total phenolic contents of the dichloromethane fraction was significantly lower than that of the ethyl acetate fraction [19], These results indicated that phenolic compounds in this species maybe have good solubility in medium polar solvents, such as water-saturated ethyl acetate [8,20].

3.2. The elucidation of compounds 1–3

The structures of the three compounds and kaempferol were shown in Fig. 1. Among the three compounds, compound **1** was isolated from the dichloromethane fraction, compound **2** and **3** were isolated from the ethyl acetate fraction. And all of the three compounds were reported in CNC flowers for the first time.

Compound **1** (Fig. 1A), Kaempferol 3-O-[2,3,4-Tri-O-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-O-acetyl-α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside. Yellow amorphous powder. $C_{43}H_{50}O_{24}$. ESI-MS, *m/z* 949.16 $[M-H]^-$. ^{1}H-NMR (500 MHz, CD_3OD) δ 0.77 (3 H, d, *J* = 6.5 Hz, H-6‴), 1.13 (3 H, d, *J* = 6.5, H-6⁗), 1.95 (1 H, s, H_{Me}-4‴), 1.96 (1 H, s, H_{Me}-2⁗), 2.05 (1 H, s, H_{Me}-2″), 2.12 (1 H, s, H_{Me}-4⁗), 2.17 (1 H, s, H_{Me}-3⁗), 3.35–3.50 (6 H, m, H-3″, 4″, 5″, 2″, 6″, 5‴), 3.80 (1 H, dd, *J* = 1.5, 11.0 Hz, H-3‴), 3.92–3.95 (2 H, m, H-6″, 5⁗), 4.60 (1 H, s, H-2‴), 4.68 (1 H, s, H-1‴), 4.94 (1 H, m, H-1⁗), 5.00–5.06 (3 H, m, H-4‴, 4⁗, 2⁗), 5.11 (1 H, m, H-3⁗), 5.48 (1 H, d, *J* = 7.5 Hz, H-1″), 6.21 (1 H, d, *J* = 2.0 Hz, H-6), 6.38 (1 H, d, *J* = 2.0 Hz, H-8), 6.89 (2 H, d, *J* = 9.0 Hz, H-3′, 5′), 8.00 (2 H, d, *J* = 9.0 Hz, H-2′, 6′). ^{13}C-NMR (125 MHz, CD_3OD) δ 179.5 (C-4), 172.3, 20.7 (AcO-C-4⁗), 172.0, 20.8 (AcO-C-4‴), 171.9, 20.8 (AcO-C-2‴), 171.9, 20.9 (AcO-C-2⁗), 171.6, 21.1 (AcO-C-3⁗), 167.0 (C-7), 163.1 (C-5), 161.6 (C-4′), 158.9 (C-9), 158.5 (C-2), 135.0 (C-3), 132.3 (C-2′, C-6′), 122.9 (C-1′), 116.3 (C-3′, C-5′), 105.7 (C-10), 103.3 (C-1″), 100.0 (C-1⁗), 100.0 (C-6), 99.0 (C-1‴), 94.9 (C-8), 78.1 (C-5″), 76.7 (C-3″), 76.0 (C-3‴), 75.4 (C-2″), 73.9 (C-4‴), 72.7 (C-2‴), 72.1 (C-4⁗), 71.2 (C-2⁗), 71.1 (C-3⁗), 70.1 (C-4″), 68.5 (C-5⁗), 67.6 (C-6″), 67.3 (C-5‴), 17.7 (C-6⁗), 17.3 (C-6‴) [21].

Compound **2** (Fig. 1B), Kaempferol 3-O-[2,3,4-Tri-O-acetyl-α-L-rhamnopyranosyl-(1→3)-4-O-acetyl-α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside. Yellow amorphous powder. $C_{41}H_{48}O_{23}$. ESI-MS, *m/z* 907.10 $[M-H]^-$. ^{1}H-NMR (500 MHz, CD_3OD) δ 1.00 (3 H, d, *J* = 5.0 Hz, H-6‴), 1.09 (3 H, d, *J* = 5.0 Hz, H-6⁗), 1.93 (3 H, s, H_{Me}-4‴), 2.06 (3 H, s, H_{Me}-2⁗), 2.08 (3 H, s, H_{Me}-4⁗), 2.13 (3 H, s, H_{Me}-3⁗), 3.35–3.50 (6 H, m, H-3″, 4″, 5″, 2″, 6″, 5‴), 3.75 (1 H, dd, *J* = 3.5, 8.5 Hz, H-3‴), 3.81–3.92 (3 H, m, H-6″, 2‴, 5⁗), 4.49 (1 H, s, H-1‴), 4.95 (1 H, d, *J* = 8.5 Hz, H-

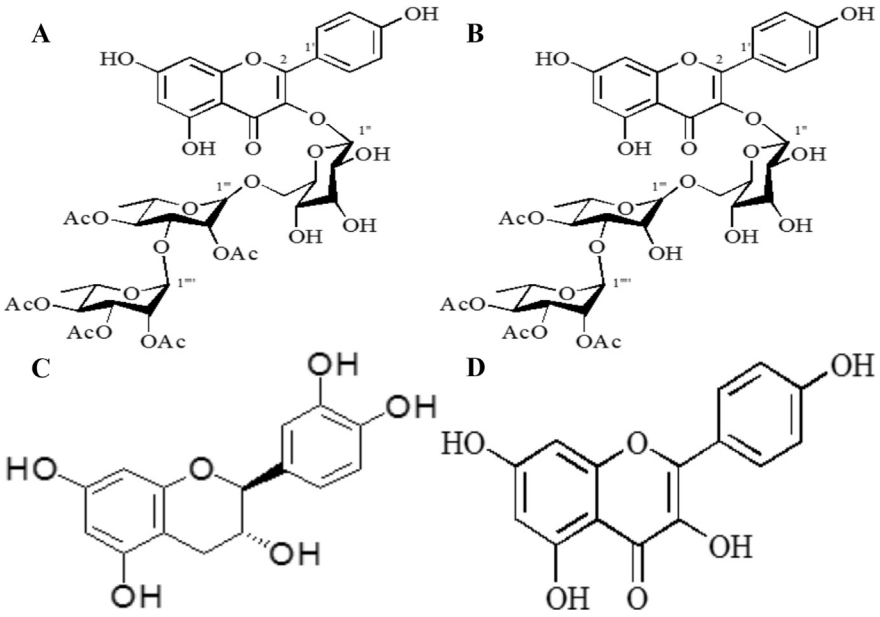

Fig. 1 – The chemical structures of compound 1 (A), compound 2 (B), compound 3 (C), and kaempferol (D).

1‴′), 5.02–5.08 (3 H, m, H-4‴, 4⁗, 2⁗′), 5.15 (1 H, m, H-3⁗′), 5.28 (1 H, dd, J = 1.5, 3.0 Hz, H-1″), 6.21 (1 H, d, J = 2.0 Hz, H-6), 6.38 (1 H, d, J = 2.0 Hz, H-8), 6.86 (2 H, d, J = 9.0 Hz, H-3′, 5′), 8.04 (2 H, d, J = 9.0 Hz, H-2′, 6′). ^{13}C-NMR (125 MHz, CD_3OD) δ 179.1 (C-4), 171.7, 20.4 (AcO-C-2⁗′), 171.7, 20.5 (AcO-C-4‴), 171.5, 20.5 (AcO-C-4‴′), 171.5, 20.6 (AcO-C-3‴′), 165.7 (C-7), 162.8 (C-5), 161.2 (C-4′), 159.2 (C-9), 158.3 (C-2), 135.2 (C-3), 132.1 (C-2′, C-6′), 122.7 (C-1′), 115.8 (C-3′, C-5′), 105.6 (C-10), 104.2 (C-1″), 100.3 (C-1⁗′), 99.8 (C-6), 99.4 (C-1‴), 94.9 (C-8), 77.8 (C-5″), 77.1 (C-3″), 76.3 (C-3‴), 75.0 (C-2″), 73.5 (C-4‴), 72.6 (C-2″), 71.8 (C-4⁗′), 71.4 (C-2⁗′), 70.7 (C-3⁗′), 70.5 (C-4′), 69.7 (C-5⁗′), 68.5 (C-6″), 67.9 (C-5‴), 17.5 (C-6⁗′), 17.4 (C-6‴) [21].

Compound **3** (Fig. 1C), Catechin. Yellow amorphous powder. $C_{15}H_{14}O_6$. ESI-MS, m/z 289.01 $[M\text{-}H]^-$. ^{1}H-NMR (500 MHz, CD_3OD) δ 2.53 (1 H, dd, J = 8.0, 16.0 Hz, H-4), 2.87 (1 H, dd, J = 5.5, 16.5 Hz, H-4), 4.00 (1 H, m, H-3), 4.57 (1 H, d, J = 7.5 Hz, H-2), 5.86 (1 H, d, J = 2.5 Hz, H-6), 5.93 (1 H, d, J = 2.5 Hz, H-8), 6.73 (1 H, dd, J = 2.0, 8.0 Hz, H-6′), 6.77 (1 H, d, J = 8.0 Hz, H-5′), 6.84 (1 H, d, J = 1.5 Hz, H-2′). ^{13}C-NMR (125 MHz, CD_3OD) δ 156.6 (C-7), 156.4 (C-5), 155.5 (C-9), 148.8 (C-3′), 148.8 (C-4′), 130.8 (C-1′), 118.8 (C-6′), 114.8 (C-5′), 113.9 (C-2′), 99.5 (C-10), 95.0 (C-6), 94.2 (C-8), 81.4 (C-2), 67.4 (C-3), 27.1 (C-4) [22].

3.3. *Antiglycation assay in BSA-glucose model*

Fig. 2A displays the inhibiting effects on AGE formation of the five fractions in this model. Their effects were concentration-dependent. Interestingly the ethyl acetate fraction could inhibit the formation of AGEs more significantly comparing with other fractions, with the percentage inhibition of 90.49% at 5 mg/mL and 32.05% at 0.2 mg/mL, and the water fraction had the least effects. The effects of the n-butanol fraction were similar to that of the ethanolic extract. The results suggested that inhibiting AGE formation was consistent with the total phenolic contents of the four fractions, except for the dichloromethane fraction. Previous researches reported that the major mechanism for phenolic compounds to inhibit AGE formation was scavenging reactive carbonyl species [6,8]. Interestingly, the total phenolic contents of the dichloromethane fraction were much lower than that of the ethanolic extract and n-butanol fraction, but its inhibiting effect was similar to theirs. It suggested that the dichloromethane fraction included some non-phenolic compounds, which could inhibit the formation of AGEs. In some previous studies, some non-phenolic compounds, could scavenge reactive carbonyl species to inhibit the AGE formation [23]. And *in vivo*, every stage of the AGE formation process might have different inhibiting mechanisms with different inhibitors [7,24].

As shown in Fig. 2B, all the three compounds, isolated from CNC flowers, had the inhibiting effects on AGE formation in this model. And the inhibiting effects became more pronounced with the increasing of the concentration. Some previous studies showed that flavonoid compounds had the inhibiting effects on the formation of AGEs [25–27]. By contrast, compound **1** showed stronger activity of the inhibiting effects on the AGE formation than that of the dichloromethane fraction. So it indicated that as one of the main components, compound **1** also might be the major active component of the dichloromethane fraction in BSA-glucose analysis. Similarly, compound **2** also might be the major active component of the ethyl acetate fraction in BSA-glucose analysis. But compound **3** showed weaker inhibiting effects than that of the ethyl acetate fraction, so it indicated that compound **3** was one major component, but not the major active component of the ethyl acetate fraction in BSA-glucose analysis. Interestingly, comparing compound **3** with kaempferol, kaempferol showed more remarkable inhibiting effects than compound **3**. The results suggested that there was one potential structure-activity relationship between the kaempferol and compound **3**, and it was similar to the publication, which showed that kaempferol had the stronger activity in BSA-glucose model [28]. Then comparing the inhibiting effects

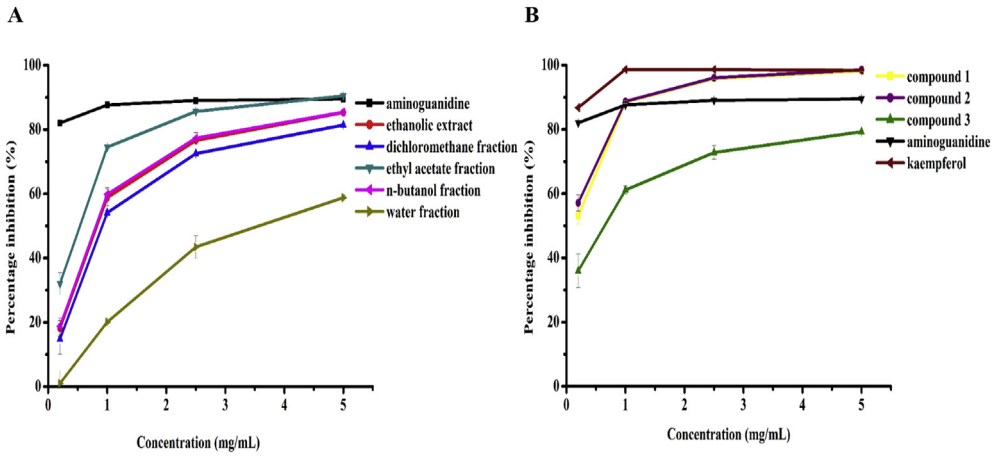

Fig. 2 – Effects on the AGE formation in BSA-glucose assay of aminoguanidine, the five fractions of CNC flowers (A) and the three compounds isolated from CNC flowers, kaempferol (B). Each value was expressed as mean ± SD (n = 3).

of compound **1**–**2** and kaempferol, kaempferol showed higher activity than compound **1**–**2**. The results suggested that the glycosides decreased the inhibiting effects, and the acetyl (on position 2‴) had no obvious significance on the inhibiting effects in this model. The study [17] supported our results, demonstrating that glycosides decreased the inhibiting effects on the AGE formation.

3.4. *Antiglycation assay in BSA-methylglyoxal model*

The results of the antiglycation assay in BSA-methylglyoxal model of the five fractions were shown in Fig. 3A. It was obvious that the inhibiting effects on AGE formation increased with concentrations, whose trend was similar to that of the BSA-glucose model. The ethyl acetate fraction, with 80.4% inhibition 5 mg/mL and 34.3% at 1 mg/mL, was more effective than the other four fractions. And the effects of inhibiting AGE formation of the ethanolic extract and n-butanol fraction were similar. The results suggested that phenolic compounds were the major factor of inhibiting AGE formation by scavenging reactive carbonyl species [7,8]. Interestingly, even though the total phenolic contents of the dichloromethane fraction was less than that of the ethanolic extract and the n-butanol fraction, they showed the similar effect on inhibiting AGE formation in BSA-methylglyoxal model (50.73% at 5 mg/mL and 17.61% at 1 mg/mL). It suggested that some non-phenolic compounds could inhibit AGE formation [23,24]. The water fraction showed almost no inhibiting effect on the formation of AGEs. These results showed that different fractions had different inhibiting effects on the AGE formation with different inhibiting mechanisms [7].

In BSA-methylglyoxal model, the compound **1**–**3** and kaempferol all had remarkable and concentration-dependent inhibiting effects (Fig. 3B). And it was reported that kaempferol, catechin and other kinds of flavonoid, flavonoid glycoside, which were found in CNC leaves and other plants, could inhibit the formation of AGEs [8,10,29]. By contrast, the inhibiting effects on AGE formation of compound **1** was stronger than that of the dichloromethane fraction, and compound **2**, **3** both showed stronger activity than the ethyl acetate fraction in the BSA-methylglyoxal model. So as the main components, compound **1** also might be the major active

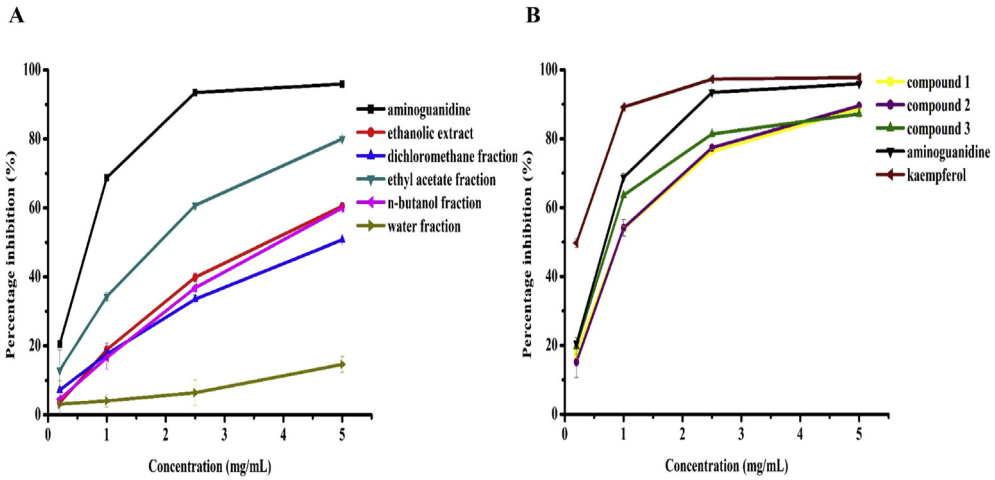

Fig. 3 – Effects on the AGE formation in BSA-methylglyoxal assay of aminoguanidine, the five fractions of CNC flowers (A) and the three compounds isolated from CNC flowers, kaempferol (B). Each value was expressed as mean ± SD (n = 3).

component of the dichloromethane fraction and compound **2**, **3** also might be the major active components of the ethyl acetate fraction in the BSA-methylglyoxal model. And kaempferol showed significant inhibiting effects, comparing with compound **1**–**3**, due to the difference between the structures [28]. A previous publication showed that the inhibiting effects of flavones were stronger than those of corresponding flavonols, flavanones [16]. And compound **1**–**2** both showed weaker inhibiting effects than keampferol indicating that the glycosides could reduce the activity of flavonoids in this model [17]. Interestingly, compound **1** and **2** had no obvious significance of the inhibiting effects on the AGE formation, and the two compounds had the same structure except a acetyl (on position 2‴), so it suggested that the acetyl (on position 2‴) had no detectable effect on the inhibition of the formation of AGEs of the flavonoid glycosides [30].

3.5. *Methylglyoxal scavenging assay*

The methylglyoxal scavenging curves of the five CNC flowers fractions were demonstrated in Fig. 4A. We found that methylglyoxal degraded with different fractions and aminoguanidine more dramatically than that without any other compounds in phosphate buffer, so all fractions had a significant effect on trapping methylglyoxal, especially the dichloromethane fraction. For the dichloromethane fraction, after incubated for 2 and 6 h, methylglyoxal remained 15.7% and 9.8%, respectively. The results suggested that some non-phenolic compounds showed acute effect on the methylglyoxal scavenging [23,24]. After incubation with the ethanolic extract, ethyl acetate and n-butanol fractions for 2 h, respectively, the methylglyoxal content was decreased with 51.0%, 31.2% and 55.9% remained, respectively, and 17.8%, 12.0% and 17.6% remained, respectively for incubating 12 h. These data showed that some phytochemicals could trap methylglyoxal effectively, especially the phenolic compounds in the ethyl acetate fraction [7,8,12]. And for the water fraction, the methylglyoxal remained 63.4% and 13.6% respectively, with incubation for 2 h and 12 h. It seemed that different phytochemicals showed different inhibiting effects due to the different inhibiting mechanisms or different scavenging rate [31].

As shown in Fig. 4B, compound **1**–**3** and keampferol all showed the ability of scavenging methylglyoxal. As the natural inhibitors against the formation of AGEs, flavonoid glycosides and flavanol had the activity of scavenging methylglyoxal [32]. Compound **1**–**2** and keampferol had the similar trendline of scavenging methylglyoxal, so it indicated that the glycosides and acetyl (on position 2‴) had no influence on the ability of scavenging methylglyoxal. Compared to keampferol, compound **3** could rapidly scavenge methylglyoxal, and after 0.5 h, 37.4% of methylglyoxal remained in the buffer, 81.0% of methylglyoxal remaining for keampferol at the same time, and before 4 h, compound **3** could scavenge methylglyoxal more rapidly than the other two compounds and keampferol. But after 4 h, the remaining methylglyoxal for keampferol was less than that of catechin, and after 12 h, 10.5% of methylglyoxal remained in the buffer for keampferol, but 23.2% of methylglyoxal remained for catechin at the same time. Kaempferol and catechin both belonged to flavonoids, and the hydroxy on B ring and/or the double bond between C-2 and C-3 might be the major factor causing the difference of ability. It was reported that the A ring was the major active site of flavonoids to scavenge methylglyoxal, especially hydroxyl group at C-5 on the A ring could enhance the ability and the double bond between C-2 and C-3 could increase methylglyoxal scavenging activity. In addition, the number of hydroxyl groups on the B ring had no significant influence on the ability of methylglyoxal scavenging [33].

3.6. *HPLC-ESI-MS/MS Identification of catechin-carbonyl adducts*

As catechin could scavenge methylglyoxal most rapidly before 4 h, catechin was incubated with methylglyoxal at 37 °C water bath for 4 h, then the reaction mixture was analyzed by HPLC-ESI-MS/MS. The results showed that some new peaks appeared in the HPLC chromatogram (Fig. 5A). The peak at 6.3 min showed the molecular ion *m/z* 289.1 $[M\text{-}H]^-$, which was the molecular ion peak of catechin. The peak at 11.7 min gave *m/z* 361.1 $[M\text{-}H]^-$, which was 72 mass units more than that of catechin, so it was identified as a monocatechin-monomethylglyoxal adduct (Fig. 5B). Similarly, The peak at 9.6 min yielded *m/z* 433.1 $[M\text{-}H]^-$, which was 144 mass

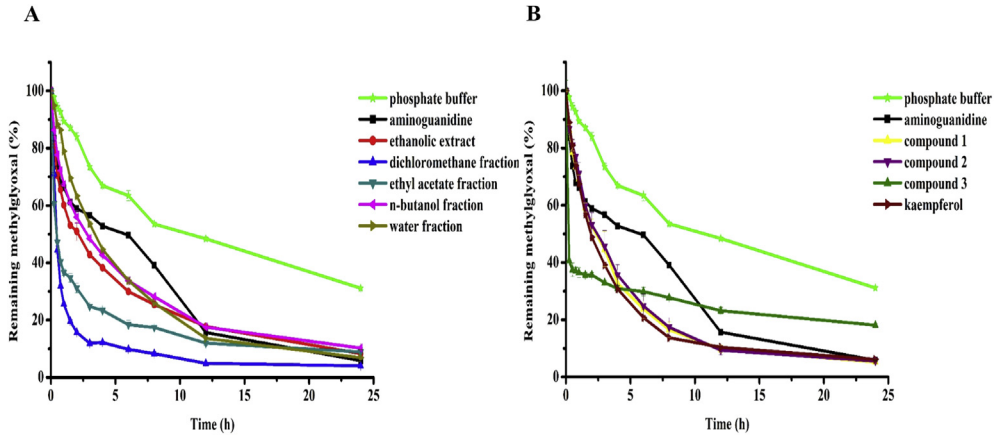

Fig. 4 – The capacity to scavenge methylglyoxal for 0-24 h at 37 °C of aminoguanidine, the five fractions of CNC flowers (A) and the three compounds isolated from CNC flowers, kaempferol (B). Each value was expressed as mean ± SD (n = 3).

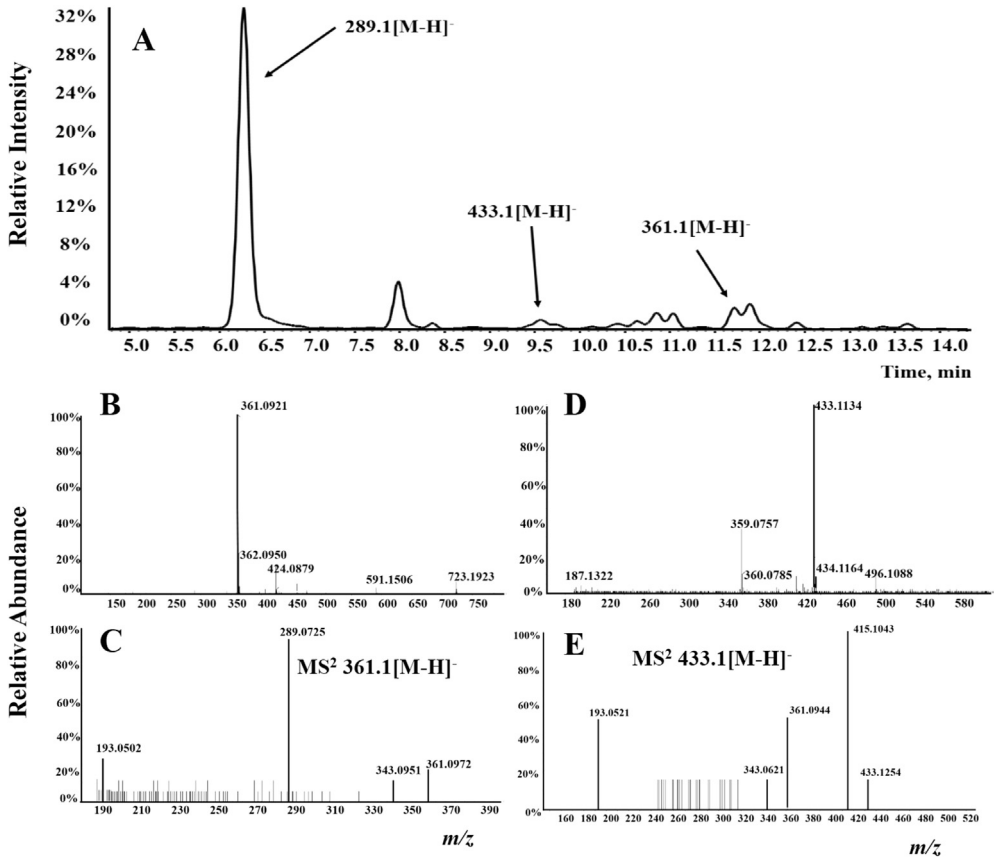

Fig. 5 – HPLC chromatogram of the mixture of catechin and methylglyoxal incubated at pH 7.4 in 37 °C water bath for 4 h (A); MS of monocatechin-monomethylglyoxal (B), MS^2 of monocatechin-monomethylglyoxal (C), MS of monocatechin-dimethylglyoxal (D), MS^2 of monocatechin-dimethylglyoxal (E) adducts.

units greater than that of catechin and identified as a monocatechin-dimethylglyoxal adduct (Fig. 5D). In the MS spectra, the *m/z* 361 $[M-H]^-$ ion underwent collision-induced dissociation MS^2 to form *m/z* 343 $[M-H-18]^-$, 289 $[M-H-72]^-$, 193 $[M-H-18-150]^-$ (Fig. 5C), and the *m/z* 433 $[M-H]^-$ ion underwent collision-induced dissociation MS^2 to form *m/z* 415 $[M-H-18]^-$, 361 $[M-H-72]^-$, 343 $[M-H-18-72]^-$, 193 $[M-H-18-72-150]^-$ (Fig. 5E). The results indicated that catechin and methylglyoxal formed the adducts through the A ring of catechin [34]. Catechin belongs to the flavanoids, which were consisted of two hydroxy-substituted aromatic rings joined by a three-carbon link, and the C-6 or C-8 of the A ring was the reactive position with methylglyoxal [34].

The phenolic compounds in different fractions of CNC flowers showed inhibiting effects on the formation of AGEs. Among these fractions, the ethyl acetate fraction showed the highest total phenolic contents and inhibiting effects on AGE formation. The ethanolic extract, ethyl acetate, and n-butanol fractions showed the inhibiting effects of AGE formation were due to the phenolic compounds, which had the abilities to trap methylglyoxal. While the dichloromethane fraction showed higher inhibition of the formation of AGEs by phenolic compounds and/or some other non-phenolic compounds. Three isolated phenolic compounds showed inhibiting effects on the AGE formation through scavenging methylglyoxal. It indicated that flavonoids from *C. nitidissima* Chi flowers could inhibit the AGE formation by scavenging methylglyoxal, but the glycosides could reduce the inhibiting effects, the acetyl (at position 2‴) had no remarkable effect on the inhibiting formation of AGEs.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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REFERENCES

[1] Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. Diabetologia 2001;44:129–46.

[2] Vitek MP, Bhattacharya K, Glendening JM, Stopa E, Vlassara H, Bucala R, et al. Advanced glycation end products contribute to amyloidosis in Alzheimer disease. Proc Natl Acad Sci U S A 1994;91:4766–70.
[3] Baynes JW. The role of AGEs in aging: causation or correlation. Exp Gerontol 2001;36:1527–37.
[4] Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res 2004;63:582–92.
[5] Liu HY, Liu HW, Wang W, Khoo C, Taylor J, Gu LW. Cranberry phytochemicals inhibit glycation of human hemoglobin and serum albumin by scavenging reactive carbonyls. Food Funct 2011;2:475–82.
[6] Peng XF, Cheng KW, Ma JY, Chen B, Ho CT, Lo C, et al. Cinnamon bark proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advanced glycation endproducts. J Agric Food Chem 2008;56:1907–11.
[7] Peng XF, Zheng ZP, Cheng KW, Shan F, Ren GX, Chen F, et al. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. Food Chem 2008;106:475–81.
[8] Wang WX, Liu HY, Wang ZN, Qi J, Yuan ST, Zhang WJ, et al. Phytochemicals from Camellia nitidissima Chi inhibited the formation of advanced glycation end-products by scavenging methylglyoxal. Food Chem 2016;205:204–11.
[9] Wang W, Yagiz Y, Buran TJ, Nunes CdN, Gu LW. Phytochemicals from berries and grapes inhibited the formation of advanced glycation end-products by scavenging reactive carbonyls. Food Res Int 2011;44:2666–73.
[10] Harris CS, Beaulieu LP, Fraser MH, McIntyre KL, Owen PL, Martineau LC, et al. Inhibition of advanced glycation end product formation by medicinal plant extracts correlates with phenolic metabolites and antioxidant activity. Planta Med 2011;77:196–204.
[11] Tsuji-Naito K, Saeki H, Hamano M. Inhibitory effects of Chrysanthemum species extracts on formation of advanced glycation end products. Food Chem 2009;116:854–9.
[12] Liu HY, Gu LW. Phlorotannins from brown algae (Fucus vesiculosus) inhibited the formation of advanced glycation end products by scavenging reactive carbonyls. J Agric Food Chem 2012;60:1326–34.
[13] Balentine DA, Wiseman SA, Bouwens LC. The chemistry of tea flavonoids. Crit Rev Food Sci Nutr 1997;37:693–704.
[14] Song LX, Wang XS, Zheng XQ, Huang DJ. Polyphenolic antioxidant profiles of yellow camellia. Food Chem 2011;129:351–7.
[15] Chen GH, Yang CY, Lee SJ, Wu CC, Tzen JTC. Catechin content and the degree of its galloylation in oolong tea are inversely correlated with cultivation altitude. J Food Drug Anal 2014;22:303–9.
[16] Matsuda H, Wang T, Managi H, Yoshikawa M. Structural requirements of flavonoids for inhibition of protein glycation and radical scavenging activities. Bioorg Med Chem 2003;11:5317–23.
[17] Shimoda H, Nakamura S, Morioka M, Tanaka J, Matsuda H, Yoshikawa M. Effect of cinnamoyl and flavonol glucosides derived from cherry blossom flowers on the production of advanced glycation end products (AGEs) and AGE-induced fibroblast apoptosis. Phytother Res 2011;25:1328–35.
[18] Chen YY, Huang YL, Wen YX. Advance in study on Chemical Constituents and Pharmacological Action of Camellia chrysantha. Guangxi Tropical Agriculture 2009;1:14–6.
[19] Xiao Y, Rui X, Xing GL, Wu H, Li W, Chen XH, et al. Solid state fermentation with *Cordyceps militaris* SN-18 enhanced antioxidant capacity and DNA damage protective effect of oats (*Avena sativa* L.). J Funct Foods 2015;16:58–73.
[20] Jang DS, Park EJ, Kang YH, Hawthorne ME, Vigo JS, Graham JG, et al. Potential cancer chemopreventive flavonoids from the stems of Tephrosia toxicaria. J Nat Prod 2003;66:1166–70.
[21] Teng XF, Yang JY, Yang CR, Zhang YJ. Five New Flavonol Glycosides from the Fresh Flowers of Camellia reticulata. Helv Chim Acta 2008;91:1305–12.
[22] Davis AL, Cai Y, Davies AP, Lewis JR. ^{1}H and ^{13}C NMR assignments of some green tea polyphenols. Magn Reson Chem 1996;34:887–90.
[23] Rahbar S, Figarola JL. Novel inhibitors of advanced glycation endproducts. Arch Biochem Biophys 2003;419:63–79.
[24] Zeng JM, Davies MJ. Evidence for the formation of adducts and S-(carboxymethyl)cysteine on reaction of alpha-dicarbonyl compounds with thiol groups on amino acids, peptides, and proteins. Chem Res Toxicol 2005;18:1232–41.
[25] Kim JM, Lee YM, Lee GY, Jang DS, Bae KH, Kim JS. Constituents of the roots of Pueraria lobata inhibit formation of advanced glycation end products (AGEs). Arch Pharm Res 2006;29:821–5.
[26] Dearlove RP, Greenspan P, Hartle DK, Swanson RB, Hargrove JL. Inhibition of protein glycation by extracts of culinary herbs and spices. J Med Food 2008;11:275–81.
[27] Bhaskar JJ, Shobha MS, Sambaiah K, Salimath PV. Beneficial effects of banana (Musa sp. var. elakki bale) flower and pseudostem on hyperglycemia and advanced glycation end-products (AGEs) in streptozotocin-induced diabetic rats. J Physiol Biochem 2011;67:415–25.
[28] Wu CH, Yen GC. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. J Agric Food Chem 2005;53:3167–73.
[29] Kim HY, Moon BH, Lee HJ, Choi DH. Flavonol glycosides from the leaves of Eucommia ulmoides O. with glycation inhibitory activity. J Ethnopharmacol 2004;93:227–30.
[30] Jang DS, Lee YM, Jeong IH, Kim JS. Constituents of the flowers of Platycodon grandiflorum with inhibitory activity on advanced glycation end products and rat lens aldose reductase in vitro. Arch Pharm Res 2010;33:875–80.
[31] Lo CY, Hsiao WT, Chen XY. Efficiency of trapping methylglyoxal by phenols and phenolic acids. J Food Sci 2011;76:90–6.
[32] Peng XF, Ma JY, Chen F, Wang MF. Naturally occurring inhibitors against the formation of advanced glycation end-products. Food Funct 2011;2:289–301.
[33] Shao X, Chen HD, Zhu YD, Sedighi R, Ho CT, Sang SM. Essential structural requirements and additive effects for flavonoids to scavenge methylglyoxal. J Agric Food Chem 2014;62:3202–10.
[34] Totlani VM, Peterson DG. Epicatechin carbonyl-trapping reactions in aqueous maillard systems: Identification and structural elucidation. J Agric Food Chem 2006;54:7311–8.