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The evaluation of catechins reducing heterocyclic aromatic amine formation: Structure-activity relationship and mechanism speculation

Ruiwei Xie [a], Haolin Zhang [b,a,1], Xiaomei Lv [a], Qiuyi Lin [a], Bing-Huei Chen [c], Yu-Wen Lai [c], Lei Chen [a], Hui Teng [a], Hui Cao [a,*]

[a] College of Food Science and Technology, Guangdong Ocean University, Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety, Guangdong Province Engineering Laboratory for Marine Biological Products, Zhanjiang, 524088, China
[b] Institute of Chinese Medical Sciences, University of Macau, Macao, China
[c] Department of Food Science, Fu Jen Catholic University, New Taipei City, 24205, Taiwan, China

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ABSTRACT

The favorable inhibitory effect of tea polyphenols on heterocyclic aromatic amines (HAAs) has been confirmed in many past studies. The objective of this study was to investigate the structure-activity relationship of catechins that act as inhibitors of HAA formation in chemical models. Two kinds of quantitative structure-activity relationship models for catechin-inhibiting-HAA were established. We chose two kinds of HAAs including 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), and five catechins including epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), epicatechin (EC), and catechin (C). The inhibitory effect of five catechins were in the following order: EGCG > ECG > EGC > C > EC. Thereinto, EGCG and ECG showed dramatically better inhibition on the formation of PhIP and MeIQx, especially EGCG. Further, the mechanisms of catechin-inhibiting-HAA were speculated by correlation analysis. The free radical-scavenging ability was predicted to be the most relevant to the inhibitory effect of ECG, EGC, EC and C on HAAs. Differently, the phenylacetaldehyde-trapping ability might be the more important mechanism of EGCG inhibiting PhIP in chemical model system. This study may bring a broader idea for controlling the formation of HAAs according to the structure of catechins.

1. Introduction

Heterocyclic aromatic amines (HAAs) are a class of compounds, containing heterocyclic rings with one or more nitrogen atoms and nitrogen containing groups within their structure (Zamora and Hidalgo, 2020). It is mainly produced by various reactions between amino acids and sugars during baking, roasting or frying foods rich in these substances. So far more than 30 varieties of HAAs have been isolated from various food product (Cao et al., 2020; Zhang et al., 2024).

As two typical thermic HAAs, 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) have gained a lot of attentions over recent years. Simultaneously, the means of reducing HAAs in thermal processing to improve human health have been extensively studied. Changing processing methods and adding food additives like antioxidants are considered effective ways (Gibis, M, 2016; Zeng et al., 2016). Benefited by the strong antioxidant activities, natural polyphenols from plants (Zhao et al., 2020) exhibited excellent inhibitory effect on the formation of HAAs during thermal processing (Kondjoyan et al., 2016). In addition, several studies have identified tea polyphenols, especially catechins, as effective natural inhibitors of PhIP and MeIQx formation (Weisburger, et al., 1994; Oguri et al., 1998).

Catechins are a class of flavan-3-ol, along with flavonols, flavanones, flavanonols, leucoanthocyanidins (flavan-3,4-diols) and anthocyanidins, belong to the class of flavonoids, a complex class of phenolic substances (Braicu et al., 2013; Sagar N A et al., 2022). Accounting for about 60%–80% on the total tea polyphenols, catechins are important functional components in tea and possess various bioactivities, such as

* Corresponding author.
E-mail addresses: xieruiwei2333@163.com (R. Xie), yc07501@umac.mo (H. Zhang), lxm030611@163.com (X. Lv), Linqiuyi202305@163.com (Q. Lin), 002622@mail.fju.edu.tw (B.-H. Chen), linda871123@gmail.com (Y.-W. Lai), chenlei841114@hotmail.com (L. Chen), tenghui850610@126.com (H. Teng), hui_cao0830@yahoo.com (H. Cao).
[1] These authors contributed equally to this work.

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antioxidant, anti-inflammatory, anti-microbial antiviral and immunomodulatory effects (Anandh Babu and Liu, 2008; Maheshwari S et al., 2022; Xiang et al., 2023). Catechins mainly consist of catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), catechin gallate (CG), epicatechin gallate (ECG), gallocatechin gallate (GCG) and epigallocatechin gallate (EGCG). Thereinto, the EGCG is the most abundant catechin, and show more impressive bioactivities. By preadding 0.04 mmol into the chemical model system containing creatinine, glucose, and phenylalanine, (Cheng et al., 2007) found that EGCG, ECG and EGC clearly suppressed the formation of PhIP. Besides, EGCG and ECG had significantly greater inhibiting activity than EGC.

The previously proposed inhibitory mechanisms mainly were attributed to the high free radical-scavenging ability of catechins, which could hinder the formation reaction of HAAs (I. Quelhas et al., 2010). The ring B is considered as the main active site of catechins antioxidant (Wang et al., 2017). Besides, catechins could also eliminate HAAs by forming HAA-catechin adducts, or reduce HAAs by forming adducts of HAA precursors and catechins. Cheng et al. (2007) suggested that radical-scavenging activity of these polyphenols was an effective mechanism of intervention, but maybe not the principal one or not be the rate-limiting step for the formation of PhIP. Cheng et al. (2009) reported that EGCG effectively inhibited the formation of PhIP and reduced the content of its key intermediate, phenylacetaldehyde. Zhu et al. (2016) found that flavonoids may inhibit PhIP formation mainly via trapping phenylacetaldehyde, instead of their free radical-scavenging abilities.

Chemical model system is an effective tool to study the formation and inhibition mechanisms of HAAs, which could exclude some complex side reactions such as Maillard reaction, caramelization reaction, etc. The chemical models of one or more specific HAAs could be established by changing the precursors and reactants (Osawa, 1992; Cheng et al., 2009). In general, it is a useful way to study the effects of different additives and variables on the formation of HAAs without using meat. In addition, chemical model is suitable for studying the structure-activity relationship of polyphenols inhibiting the formation of HAAs. Some formation and inhibition mechanisms of HAAs were also demonstrated using chemical model systems (Ren et al., 2020; Jing et al., 2022).

Significantly, the current study on the relationship between catechin monomers of different structures and the inhibitory effects of HAAs and the related mechanisms are not comprehensive. In this study, five catechin monomers (EC, C, ECG, EGC, EGCG) showed in Fig. 1 and two typical thermic HAAs (PhIP and MeIQx) were taken as objects to investigate the special role of the different active structural groups of catechins for inhibiting the formation of PhIP and MeIQx in chemical model system using Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). Moreover, the mechanisms of catechins inhibiting HAAs were demonstrated in many ways.

2. Methods and materials

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO) was acquired from Alfa Aesar Chemical Co., Ltd. L-phenylalanine (99%), L-threonine (99%) and creatinine were purchased from J&K Scientific, ECG (98%) and C (98%) were obtained from Chroma Biotechnology Co., Ltd. EGCG (98%), EGC (98%), EC (97%) and D-(+)-glucose were obtained from Aladdin Industrial Corporation. 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP, CAS: 105650-23-5), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx, CAS: 77500-04-0), 1-Methyl-9H-pyrido[3,4-b]indole (Harman, CAS: 486-84-0) standard was obtained from TCI (Shanghai) Chemical Industry Development Co., Ltd. Distilled water (>99.9%) was provided by Watsons Water (Hong Kong, China). Acetonitrile (MS grade) and formic acid (>99.9%) was obtained from Thermo Fisher Scientific (Bangkok, Thailand). Methanol (LC-MS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid was provided by Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Anhydrous ethanol was purchased from Xilong Scientific (Guangzhou, China).

2.2. UPLC-MS/MS condition

PhIP and MeIQx were analyzed on a Waters ACQUITY UPLC H-Class System with an PDA Detector (Waters, MA, USA) using a ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm, SKU: 186002352), with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) as a mobile phase. The total flow was 0.3 mL/min, with a following gradient elution: 0–2 min, 5% B; 2–9 min, 5%–50% B; 9–13.5 min, 50%–95% B; 13.5–14.5 min, 95%–50% B; 14.5–16 min, 50%–5% B; 16–21 min, 5% B. The column oven temperature was set at 35 °C. The flow rate was set at

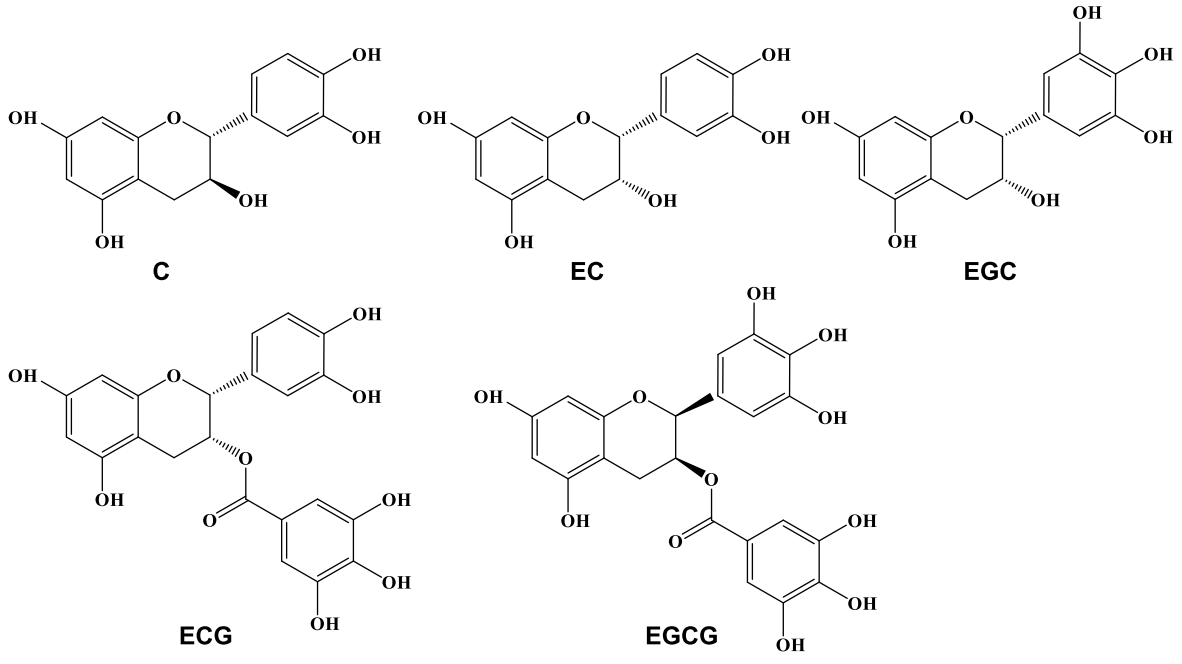

Fig. 1. The structure of five kinds of catechin monomers used in our study.

0.3 mL/min. Samples were injected in a volume of 1 μL.

MS detection was performed on a Waters XEVO G2-XS QTOF combined with an ESI source (Waters, MA, USA). The MSE Centroid mode was used for acquirement of data under sensitive and positive ion mode (ESI+) over a scan range of 500–1200 m/z, with a scan time of 0.5 s. The parameters of the LockSpray ion source were set as follows: a capillary voltage of 1.5 kV, sampling cone voltage of 40 V, source offset of 80 V, source temperature of 120 °C, desolvation temperature of 450 °C, cone gas flow (N^2) of 50 L/h and desolvation gas flow (N^2) of 700 L/h. The data was processed by MassLynx V 4.1 software (Waters Corp., Milford, MA, USA).

2.3. Establishment of PhIP-producing and MeIQx-producing chemical model systems

The chemical model systems of PhIP and MeIQx were established according to the previously studies (Cheng et al., 2007; Jing et al., 2022; Oguri et al., 1998) with some minor modifications. The compositions of PhIP and MeIQx chemical model systems are shown in Table 1. The PhIP-producing chemical model system containing 10 mmol/L phenylalanine, 5 mmol/L glucose, 10 mmol/L creatinine dissolved in 5 mL water; the MeIQx-producing chemical model system containing 40 mmol/L phenylalanine, 20 mmol/L glucose, 40 mmol/L creatinine dissolved in 5 mL water. With or without the addition of catechins, the reactants of the two chemical model systems were all dissolved in distilled water and added to pressure tubes with plug. The tubes were inserted into an air oven set at 200 °C and heated for 60 min. After heating, the tubes were immediately cooled in ice. Then, the reaction solutions were centrifuged at 15,000 rpm for 5 min, and the supernatants were collected for further filtration by a 0.22-μm water phase filter. Five replicates were performed for each treatment. Finally, the treated sample solutions were stored at 4 °C for further analysis on UPLC-MS/MS.

2.4. Heating temperature and heating time on HAAs formation in PhIP and MeIQx chemical model systems

The effects of heating temperature and heating time on the formation of PhIP and MeIQx were explored on corresponding-producing chemical model systems. The mixture solutions of reactants in two chemical models (PhIP and MeIQx) were first prepared according to the details in Table 1, respectively.

To explore the effect of temperature on the formation of PhIP and MeIQx in their separate chemical model systems, six heating temperatures (120, 140, 160, 180, 200 and 220 °C) were selected. The heating time was set as 60 min. After heating, samples were treated and analyzed as described in section 2.3.

To explore the effect of heating time on the formation of PhIP and MeIQx in their separate chemical model systems, six heating time (15, 30, 45, 60, 75 and 90 min) were selected. The heating temperature was set at 200 °C. After heating, samples were treated and analyzed as that described in section 2.3.

Table 1
The compositions of PhIP and MeIQx chemical model systems.

Reactants	Chemical model systems (mM)	
	PhIP system	MeIQx system
Phenylalanine	10	–
Threonine	–	40
Creatinine	10	40
Glucose	5	20
Catechins	0.5–2.5	2–10

2.5. Investigating the different inhibition effect of five catechins on PhIP in PhIP-producing chemical model system

In addition, five catechin monomers with representative structural characteristics (EC, C, ECG, EGC, EGCG) were selected to investigate the special roles of the different active structural groups of catechins for inhibiting the formation of PhIP and MeIQx. We examined the effects of different concentrations of five catechin monomers on PhIP formation in PhIP-producing chemical model system. Considering the concentration setting, some studies showed that EGCG in molar quantity as low as one-fourth that of phenylalanine was capable of suppressing the formation of phenylacetaldehyde by nearly 90% relative to the control (Cheng et al., 2009). As the condensation reaction of phenylacetaldehyde and creatinine is the key reaction in the formation of PhIP, our study set the maximum concentration of five catechin monomers to a quarter of phenylalanine, and then reduced proportionally to explore the effect of different concentration of each catechin monomers on producing PhIP. Five catechins (EC, C, ECG, EGC, EGCG) were dissolved in DMSO and added into PhIP-producing chemical model system then mixed thoroughly, the concentrations of five catechins reached 0, 0.5, 1, 1.5, 2 and 2.5 mmol/L, respectively. When considering the heating temperature and heating time, 200 °C and 60 min was chosen. After heating, samples were treated and analyzed as described in section 2.3.

The results were expressed by the inhibition rate of five catechins to PhIP at each concentration. The half maximal inhibitory concentration (IC_{50}) was also shown, which refers to the concentration of catechin monomers when the inhibition effect on PhIP reaches 50%. The inhibition rate of all catechins to PhIP were calculated by the following formula: Inhibition rate (%) = [(1-adjusted sample HAA peak area/the average of adjusted control (0 mM) HAA peak area) × 100%]. The IC_{50} value was calculated by SPSS 19.0 software.

2.6. Investigating the different inhibition effect of five catechins on MeIQx in MeIQx-producing chemical model system

The effects of different concentrations of five catechin monomers on MeIQx formation in MeIQx-producing chemical model system were examined using the similar method in section 2.5. As explained above, five catechins were dissolved in dimethyl sulfoxide and added into MeIQx-producing chemical model system then mixed thoroughly, the concentrations of five catechins reached 0, 2, 4, 6, 8 and 10 mmol/L respectively. After heating at 200 °C for 60 min, samples were treated and analyzed as described in section 2.3. The results were also expressed by the inhibition rate of five catechins to MeIQx at each concentration as well as the IC_{50} of catechins to MeIQx.

2.7. Assessing the relationship between free radical-scavenging abilities of catechins and inhibitory effects of five catechins on HAAs (PhIP and MeIQx) by DPPH assay

Since free radical-scavenging is a widely considered mechanism for inhibiting the formation of HAAs in previous study (Wang et al., 2017). In this study, we systematically evaluated the free radical-scavenging abilities of the five catechins standards by DPPH-scavenging assay (Li, 2018), and monitored their change of heating time by DPPH antioxidant experiment. The reducing ability of antioxidants towards DPPH was evaluated by monitoring the decrease of its absorbance at 517 nm. Results were expressed as % scavenging activity of DPPH at a fixed catechin monomers concentration for all the samples.

2.7.1. The free radical-scavenging abilities of the five catechin standards

The concentration was selected for five catechins as the PhIP-producing chemical model system (0, 0.5, 1, 1.5, 2 and 2.5 mmol/L) and MeIQx-producing chemical model system (0, 2, 4, 6, 8 and 10 mmol/L). The following methods were according to the previously studies (Xiao et al., 2020; Wang et al., 2021) with some minor

modifications: 0.0039 mg DPPH was dissolved completely in 100 mL ethanol then kept in dark for 30 min 100 μL distilled water was added to 900 μL DPPH solution as the blank control, 100 μL sample was added to 900 μL DPPH solution as the sample group. The plate was kept protected from light at room temperature for 30 min, then the absorbance was recorded at 517 nm with a Varioskan Flash-Full wavelength multi-functional enzyme marker from Thermo Fisher Scientific. The inhibition ratio (% of control) was obtained.

2.7.2. The change of free radical-scavenging ability during the heating process in the catechin-inhibiting-HAA systems

The same concentration was selected for five catechins to add to the PhIP-producing chemical model system (1.5 mmol/L) and MeIQx-producing chemical model system (6 mmol/L), with the same compositions of catechin-inhibiting-PhIP system (section 2.5) and catechin-inhibit-MeIQx system (section 2.6). Then, the DPPH scavenging ability of every 15 min from 0 min to 60 min heating period was tested. Before the assay, the PhIP chemical model sample solution was diluted 10 times and the MeIQx chemical model sample solution were diluted 100 times. Samples were treated and analyzed as described in section 2.7.1.

2.8. Evaluation of phenylacetaldehyde-trapping ability in PhIP chemical model systems by gas chromatography-mass spectrometry (GC-MS)

Since phenylacetaldehyde-trapping ability might be another way to inhibit PhIP, it is necessary to compare the trapping ability of different catechins for phenylacetaldehyde, the key PhIP precursor, in the formation of PhIP. One mL of each reaction mixtures showed in 2.6 were extracted by 3 mL ethyl acetate. The ethyl acetate extract was collected, blew dry with nitrogen at 50 °C, redissolved by 200 mL methanol, filtered, and then subjected to GC-MS analysis.

The samples were analyzed on the Shimadzu GCMS-TQ8040 NX triple quadrupole gas chromatography-mass spectrometer equipped with an automatic sampler (AOC-20i). Separation was carried out on a capillary column of PEG20M. Analyses methods were as follows: inlet temperature, 50 °C; temperature program, 10 °C/min; up to 180 °C and hold for 5 min; up to 240 °C and hold for 5 min; column gas flow, 3 mL/min (N_2); injection volume, 0.8 μL. The correlation coefficient (R^2) for the phenylacetaldehyde standard curve was 0.9998.

2.9. Direct reaction between catechins and phenylacetaldehyde in diethylene glycol chemical model system

Catechins could also eliminate HAAs by forming adducts of HAA precursors and catechins. As phenylacetaldehyde-trapping ability might also be a way to inhibit PhIP, it is necessary to compare the trapping ability of different catechins for phenylacetaldehyde, the key PhIP precursor. However, due to the continuous generation and consumption of phenylacetaldehyde in the system is constantly being generated and consumed, the binding speed and amount could not be accurately determined, resulting in the inability to compare the ability of different catechins to bind phenylacetaldehyde. Therefore, a chemical model was constructed with the same concentration but only composed of phenylacetaldehyde and catechins, so that the initial amount of phenylacetaldehyde was the same as the PhIP chemical model system, and the ability of five catechins to capture the PhIP precursor phenylacetaldehyde was more clearly compared.

This reaction was according to the previous study with some minor modifications. Firstly, putting 10 mM phenylacetaldehyde and 4 mM five catechin monomers into a two-phase solution which contained 5 mL diethylene glycol. After heating, the tubes were immediately cooled in ice. After centrifuging the mixture at 15,000 rpm for 5 min at 4 °C, the supernatant was collected and stored at 4 °C. And then filtered through a 0.22 μm water phase filter, subjected to UPLC-MS/MS analysis for the phenylacetaldehyde-catechin adducts.

2.10. Correlation analysis

In this study, SPSS 19.0 software was used to conduct bivariate correlation analysis on the IC_{50} value of five catechins inhibition and their corresponding DPPH radical-scavenging rate in PhIP and MeIQx chemical model systems, as well as the IC_{50} value of five catechins inhibition and their corresponding phenylacetaldehyde-trapping ability in PhIP chemical model system. This can be intuitively stated that whether the inhibiting ability of the five catechins to PhIP and MeIQx is related to their radical-scavenging abilities and the phenylacetaldehyde-trapping abilities. The significance test was conducted by bilateral test (T), and the correlation coefficient was selected by Spearman.

2.11. Method validation

In this study, the linear range of PhIP and MeIQx of UPLC-MS/MS detection method were analyzed. PhIP and MeIQx was identified by comparing the retention time and MS spectrum with commercial standard. Peak areas were adjusted relative to the peak area of the internal standard (Harman).

Standard curve concentration gradient samples equivalent to PhIP and MeIQx concentration gradients 0.0001, 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25, 0.5 and 1 μmol/L were prepared, and then added 1 μL of Harman internal standard solution (10 mmol/L) to 200 μL of sample solution. The ratio of PhIP and MeIQx peak area to internal standard peak area (I) and drug concentration to be measured (X) in the PhIP and MeIQx standard curve sample were linearly regression and the standard curve equation was established by using the weighted least square method.

2.12. Statistical analysis

MassLynx 4.1 SCN 805 software was used to carry out the data acquisition. External calibration curves, obtained by linear regression of a plot of the standard/IS peak area HAAs ratios against the HAAs concentrations, were used to calculate the compound concentrations in the samples.

For all experiments, three replicates were set for each treatment ($n = 3$), and the data are presented as the mean ± standard deviation (SD) in tables. Line graphs and bar graphs were generated by GraphPad Prism 9. The 3D waterfall plots of stability data were analyzed and plotted by OriginPro (2019b) software (OriginLab, Northampton, Massachusetts, USA). The degree of significance was denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. The test data were analyzed by Spearman correlation using SPSS 19.0 software.

3. Results and discussion

3.1. Method validation

The linear range for PhIP was 0.0001–1 μmol/L and 0.0001–0.5 μmol/L for MeIQx, there was a high linear correlation in this linear range. Quantitative determination of PhIP was performed using a calibration curve ($Y = 558592X + 7593.4$, $R^2 = 0.992$) and MeIQx was performed using a calibration curve ($Y = 683550X + 1995.8$, $R^2 = 0.9975$) at ten calibration levels (0, 0.0001, 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25, 0.5 and 1 μmol/L). Quantification was calculated based on the peak area associated with the PhIP and MeIQx standards and the internal standard Harman.

3.2. Effect of heating temperature and heating time on HAAs formation in PhIP and MeIQx chemical model systems

To investigate the formation of PhIP and MeIQx in chemical model systems, UPLC-MS/MS analysis was performed for samples from aqueous PhIP-producing and MeIQx-producing model systems.

For heating temperature, our result showed that when the heating temperature was between 120 and 180 °C, the formation of HAAs in corresponding HAAs chemical model system were slowly growing from 0 μmol/L to 0.01 μmol/L, then reached nearly 0.03 μmol/L at 200 °C. However, when the heating temperature rose to 220 °C, the amount of PhIP rapidly rose to 0.3 μmol/L. The production of both PhIP and MeIQx continued to increase until reached the maximum (Fig. 2). That is because HAAs are extremely stable compounds that will not degrade or turn into other substances during chemical reactions within a certain temperature range. We can tell that the increase rate of PhIP was significantly better than that of MeIQx at high heating temperature in corresponding HAAs chemical model system, which was mainly related to the types of preconditions and reaction mechanisms required for the formation of different HAAs. This is consistent with previous research, the effects of temperature and time on the formation of HAAs were studied in a liquid model by Arvidsson et al. (2010).

For heating time, our result showed that when the heating time was between 15 and 60 min, PhIP grew slowly; when the heating time reached 75 min or above, the formation of PhIP began to grow rapidly in PhIP-producing chemical model system. Similarly, MeIQx grew slowly in the first 30 min, while began to grow rapidly when the heating time reached 45 min or above in MeIQx-producing chemical model system. In general, with the heating time increased, the increase rate of MeIQx was obviously better than that of PhIP in corresponding HAAs chemical model system.

In general, the content of HAAs increased with the increase of processing temperature and processing time, which was consistent with early study (P et al., 2017). In this study, we chose 200 °C and 60 min as the conditions for the establishment of the PhIP and MeIQx chemical model systems to explore the subsequent experiment on the effect of the addition of catechins on the generation of PhIP and MeIQx.

3.3. *Effect of five catechins on the formation of PhIP in PhIP-producing chemical model system*

To investigate the effect of five catechins on the formation of PhIP in chemical model system, UPLC-MS/MS analysis was performed for samples from aqueous PhIP-producing models with the addition of five catechin monomers. The results are shown in Fig. 3. When the five catechin monomers was added by one–fourth that of phenylalanine, the formation of PhIP was suppressed by nearly 80–93% relative to the control. According to the IC_{50} value, EGCG had the strongest inhibition

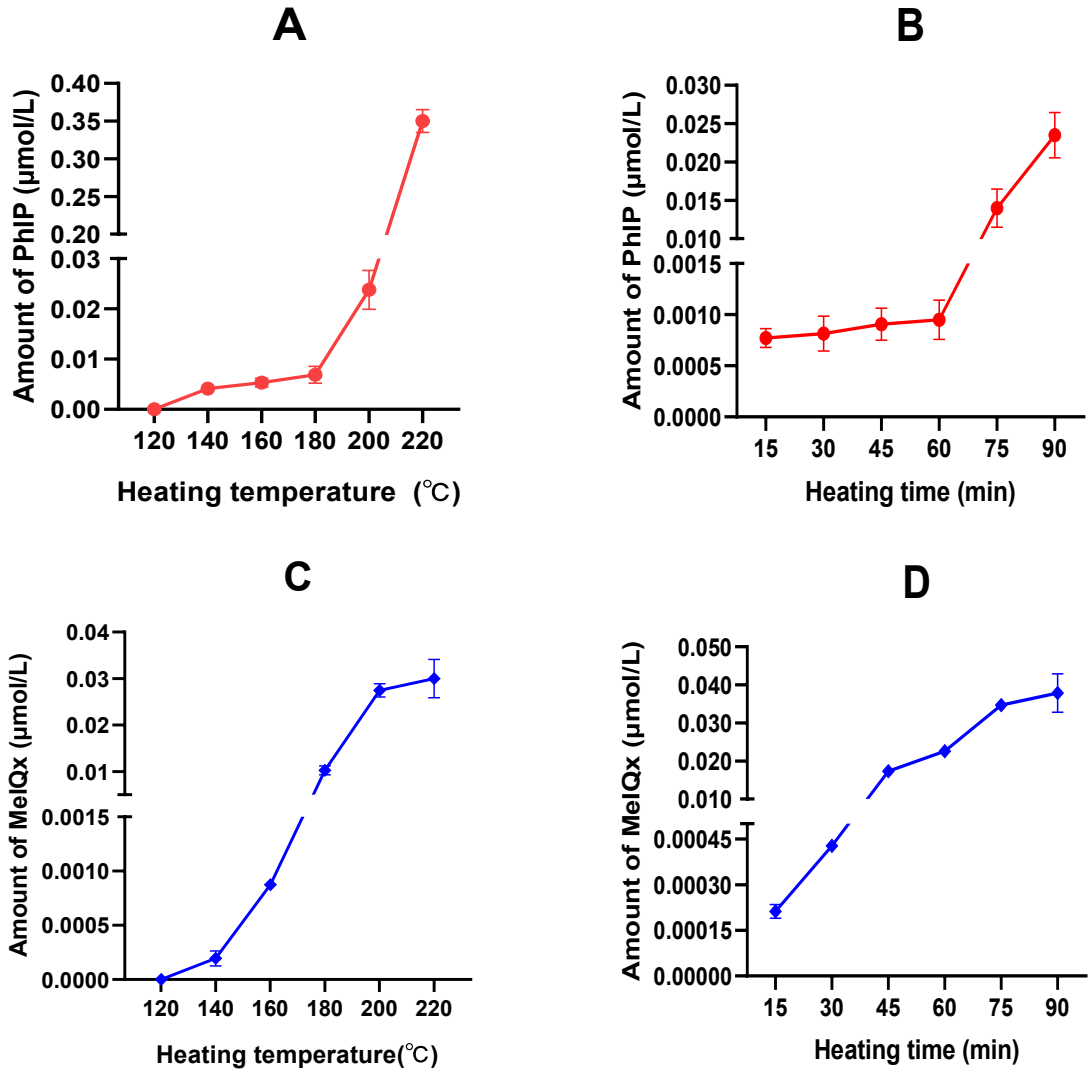

Fig. 2. The effects of different heating temperatures and times on the formation of PhIP or MeIQx in PhIP-producing or MeIQx-producing chemical model system (n = 3) (A) Different heating temperatures on the formation of PhIP. (B) Different heating time on the formation of PhIP. (C) Different heating temperatures on the formation of MeIQx. (D) Different heating time on the formation of MeIQx.

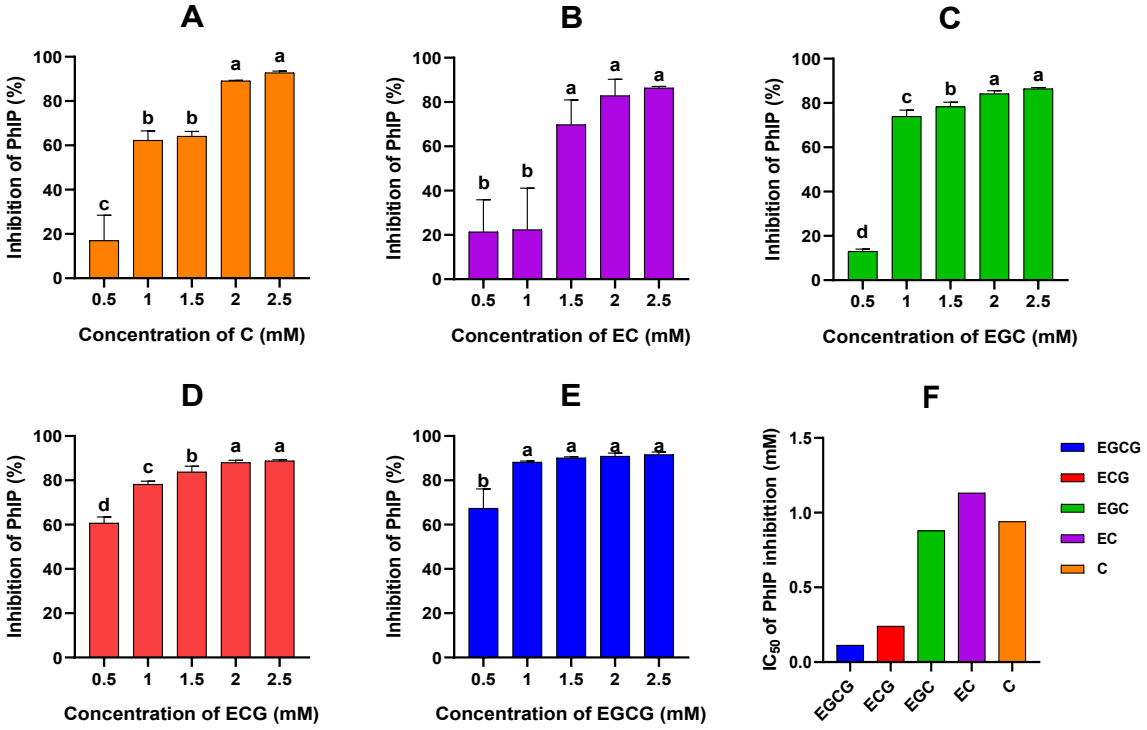

Fig. 3. The effects of five catechins on the formation of PhIP in PhIP-producing chemical model system (n = 3). (A–E) The inhibitory rate of C, EC, EGC, ECG, EGCG on the formation of PhIP. (F) The IC_{50} value of five catechins inhibiting PhIP in chemical model system.

ability, followed by ECG, EGC, C and EC in PhIP-producing chemical model system. At the lowest concentration (0.5 mmol/L), the inhibiting rate of PhIP by EGCG and ECG already reached 64% and 61%, meanwhile EGC, EC and C just reached 20% or lower. The inhibiting rate of PhIP by EC, EGC and ECG was both 86–88% at the highest concentration (2.5 mmol/L) while EGCG and C achieved 91% and 92%. At the

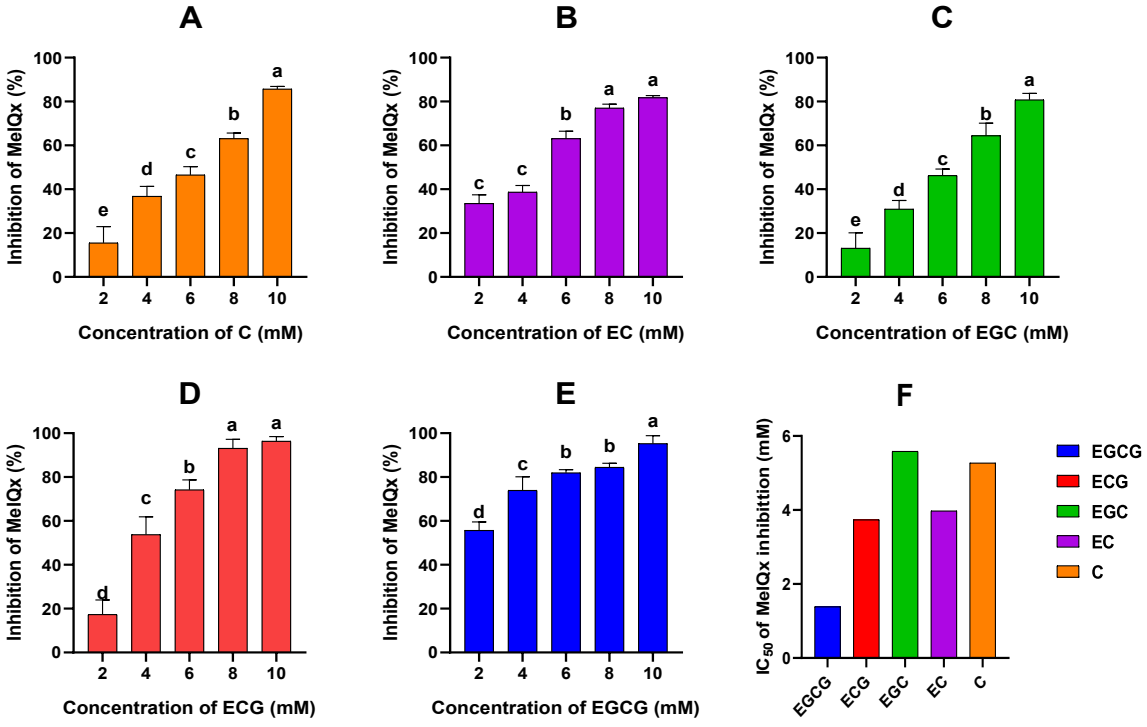

Fig. 4. The effects of five catechins on the formation of MeIQx in MeIQx-producing chemical model system (n = 3). (A–E) The inhibitory rate of C, EC, EGC, ECG, EGCG on the formation of MeIQx. (F) The IC_{50} value of five catechins inhibiting MeIQx in chemical model system.

concentration range of 1–2.5 mmol/L in PhIP chemical model system, the inhibition rate of PhIP by EGCG and ECG both could reach 80%–90%, followed by EGC reached 70%–90%, and the inhibition rate of PhIP by EC and C could not reach 80% until their concentration in PhIP chemical model system was 2.5 mmol/L. In general, As the concentration of catechins increased, the inhibitory effect was enhanced. According to the IC_{50} value, the inhibitory effect on PhIP ranged from strong to weak was EGCG > ECG > C > EGC > EC (Fig. 3).

3.4. Effects of five catechins on the formation of MeIQx in MeIQx-producing chemical model system

To investigate the effect of five catechins on the formation of MeIQx in chemical model system, UPLC-MS/MS analysis was performed for samples from aqueous PhIP-producing models with the addition of five catechin monomers. The result showed that when five catechin monomers was one–fourth that of threonine was capable of suppressing the formation of MeIQx by nearly 80–95% relative to the control (Fig. 4). According to the IC_{50} value, EGCG had the strongest inhibition ability, followed by ECG, EC, C and EGC in MeIQx-producing chemical model system. At the lowest concentration (0.5 mmol/L), the inhibiting rate of PhIP by EGCG already reached 56%, followed by 34% of EC, meanwhile ECG, EGC and C were lower than 20%. The inhibiting rate of PhIP by EC, EGC and C was both 81–85% at the highest concentration (2.5 mmol/L), while EGCG and ECG achieved 95% and 96%. In general, As the concentration of catechins increased, the inhibitory effect was enhanced. According to the IC_{50} value, the inhibitory effect on PhIP ranged from strong to weak was EGCG > ECG > EC > C > EGC (Fig. 4).

3.5. Free radical-scavenging ability of five catechins in PhIP and MeIQx chemical model systems

To investigate the contribution of the free radical-scavenging ability of the five catechins on inhibiting the formation of HAAs in chemical model systems, the antioxidant activities of these catechin monomers were evaluated by DPPH assay in our study. In general, the scavenging rate of the DPPH radical by five catechin monomers showed a gradually decreasing trend with heating time. The one with the strongest DPPH scavenging ability was EGCG, followed by ECG and EGC in both PhIP and MeIQx chemical model systems. Differently, C was the least active one in PhIP-producing system while EC was the least active one in MeIQx-producing system (Fig. 5A and B). Interestingly, the strength of DPPH radical-scavenging rate of the five catechin monomers was similar but slightly different to that of their inhibitory abilities in PhIP and MeIQx chemical model systems showed in Figs. 3 and 4. To investigate the relationship between radical-scavenging activity on PhIP and MeIQx

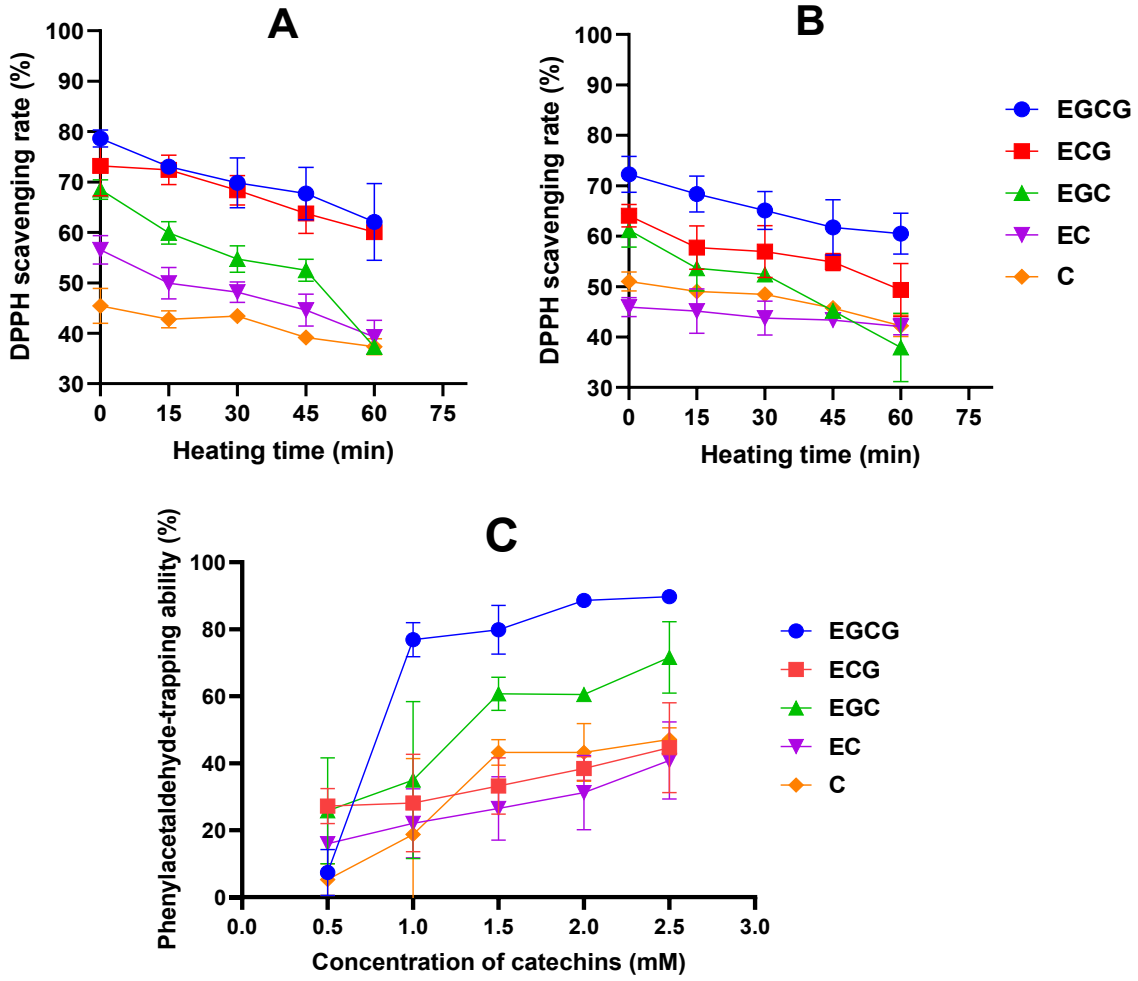

Fig. 5. Free radical-scavenging ability and phenylacetaldehyde-trapping ability of five catechins in HAA chemical model. (A) Free radical-scavenging rate of five catechins under different heating time in PhIP-producing chemical model system (n = 3). (B) Free radical-scavenging rate of five catechins under different heating time in MeIQx-producing chemical model system (n = 3). (C) Phenylacetaldehyde-trapping ability of five catechins under different concentration in PhIP-producing chemical model system (n = 3).

formation, DPPH radical-scavenging rate of five catechins and their inhibitory effects on PhIP and MeIQx formation in corresponding chemical model systems were analyzed by correlation analysis.

From the structure-activity point of view, structural features, namely the number of galloyl and hydroxyl (OH) groups in catechins molecules, play an important part in their antioxidant properties. The more hydroxyl groups the higher antioxidant activity is expected. Moreover, the presence of a second hydroxyl group on the ortho or para position is going to increase the antioxidant activity (Rice-Evans et al., 1997). Studies have shown that in vitro the catechins are effective antioxidants on a molar basis follows the order: EGCG ≈ ECG > EGC > C ≈ EC (Salah et al., 1995). Their result was similar with our result shown in Table 2, which showed that the IC_{50} of free radical-scavenging ability of the five catechins was in the following order on a molar basis: EGCG > ECG > C > EGC > EC. Such results were also consistent with the number and arrangement of phenolic hydroxyl groups. First, the hydroxyl group at the C3 position in the ring C of EGCG and ECG was replaced by an esterified gallic acid, and EGCG got one more hydroxyl group in the ring B than ECG, which made EGCG have the best radical-scavenging ability and ECG took the second place. Then, EGC was structurally similar to EC and C, with an additional hydroxyl group adjacent to the *o*-diphenolic structure in the ring B enhancing the antioxidant activity, which made EGC the third radical-scavenging ability, followed with EC and C the least active.

3.6. Relationship between free radical-scavenging ability and inhibition on HAAs formation of five catechins in PhIP and MeIQx chemical model systems

In this study, the relationship between the effects of five catechins on PhIP inhibition and the free radical-scavenging ability of five catechins in PhIP and MeIQx chemical model systems were analyzed by correlation analysis.

As shown in Table 2, the inhibitory ability of five catechin monomers on PhIP and MeIQx chemical model systems with their free radical-scavenging abilities was not exactly same. Except for the correlation of the inhibitory ability of EGCG was not significantly correlated with its free radical-scavenging ability in both PhIP and MeIQx chemical model systems, the rest of the cases the two abilities were significantly correlated. Thereinto, the correlation of the inhibitory ability of ECG in MeIQx chemical model system was significantly positively correlated with its free radical-scavenging ability at the 0.05 level (bilateral). In the rest of the cases, the two abilities were significant positive correlated at the 0.01 level (bilateral).

Poor correlations ($R^2 = 0.475$ and 0.308) were found between the inhibitory ability and free radical-scavenging ability of EGCG in both PhIP and MeIQx chemical model systems. The results showed that antioxidant activity of EGCG was not correlated with their inhibitory effects of PhIP and MeIQx formation. That is to say, high antioxidant capacity does not necessarily lead to strong inhibitory effects on HAAs formation. In other words, free radical-scavenging ability might not the main way for the inhibition of EGCG in chemical model systems. It is in agreement with previous studies which also found the poor relation between antioxidant activities and inhibition of HAAs formation (Zhu et al., 2007).

Although when EGCG is replaced by EGC, EC and C, the situation is reversed, we can still say that the capacities of catechins to inhibit HAAs formation were not completely correlated with their radical-scavenging abilities. The role of polyphenols in Maillard reaction related to HAAs formation maybe more complex than just being free radical-scavenging agents.

3.7. Phenylacetaldehyde-trapping effects of five catechins in PhIP chemical model system

Heating of phenylalanine can give rise to many degradation products, such as phenylacetaldehyde, phenylethylamine, phenylethanol, phenylacetic acid and styrol. Among them, only the first two products have been demonstrated to form PhIP by reacting with creatinine (Zchling and Murkovic, 2002). Phenylacetaldehyde, a kind of reactive carbonyl species (RCS), is the main volatile compound produced by the degradation of phenylalanine, which can react with creatinine to form PhIP (Cheng et al., 2009). Zchling and Murkovic (2002) found that no formation of PhIP occurred in a model system with creatinine and phenylalanine that was performed at room temperature for 2 weeks. This is an additional indication that the degradation of phenylalanine to phenylacetaldehyde is necessary for the formation of PhIP (Lutz et al., 1983). Therefore, our study chose phenylacetaldehyde as the target compound, then phenylacetaldehyde-trapping ability of five catechins was obtained by calculation to evaluate their HAAs inhibition ability.

As shown in Fig. 5C, in general, the phenylacetaldehyde-trapping ability by five catechin monomers showed a gradually decreasing trend with their concentration. The one with the strongest phenylacetaldehyde-trapping ability was still EGCG, just like its free radical-scavenging ability. The addition of catechins reduced the remaining amount of phenylacetaldehyde in PhIP chemical model system. The consumption of this RCS intermediate by catechins would thus greatly reduce their availability for PhIP formation, and further effectively attenuate the amount of HAAs production in the reaction system.

3.8. Relationship between phenylacetaldehyde-trapping ability and inhibition on PhIP formation of five catechins in PhIP-producing chemical model system

In this study, the relationship between the effects of five catechins on PhIP inhibition and the phenylacetaldehyde-trapping ability of five catechins was analyzed by correlation analysis.

As shown in Table 2, a good correlation ($R^2 = 0.958$) was found between the inhibitory ability and phenylacetaldehyde-trapping ability of EGCG in PhIP chemical model system, which showed that phenylacetaldehyde-trapping activity of EGCG was positively correlated with its inhibitory effects of PhIP formation. This result was agreed with

Table 2
Correlation analysis of IC_{50} values of the five catechins between their HAA-inhibiting abilities and corresponding free radical-scavenging abilities and phenylacetaldehyde-trapping abilities.

Catechins	IC_{50} of PhIP inhibition/(mmol/L)	IC_{50} of MeIQx inhibition/(mmol/L)	Free radical-scavenging ability of catechins (IC_{50} of DPPH assay)/(mmol/L)	Phenylacetaldehyde-trapping ability of catechins (IC_{50})/(mmol/L)	Correlation analysis between IC_{50} of PhIP inhibition and IC_{50} from DPPH assay	Correlation analysis between IC_{50} of MeIQx inhibition and IC_{50} of DPPH assay	Correlation analysis between IC_{50} of PhIP inhibition and Phenylacetaldehyde-trapping ability (IC_{50})
EGCG	0.11	1.39	0.07	0.90	0.475	0.308	0.958**
ECG	0.24	3.74	0.04	5.89	0.686**	0.536*	0.514*
EGC	0.97	5.59	0.82	1.27	0.911**	0.925**	0.607*
EC	1.13	3.98	1.59	6.56	0.871**	0.957**	0.546*
C	0.94	5.27	0.32	2.52	0.904**	0.961**	0.729**

*significant correlation at 0.05 level, **. Significantly correlated at 0.01 level.

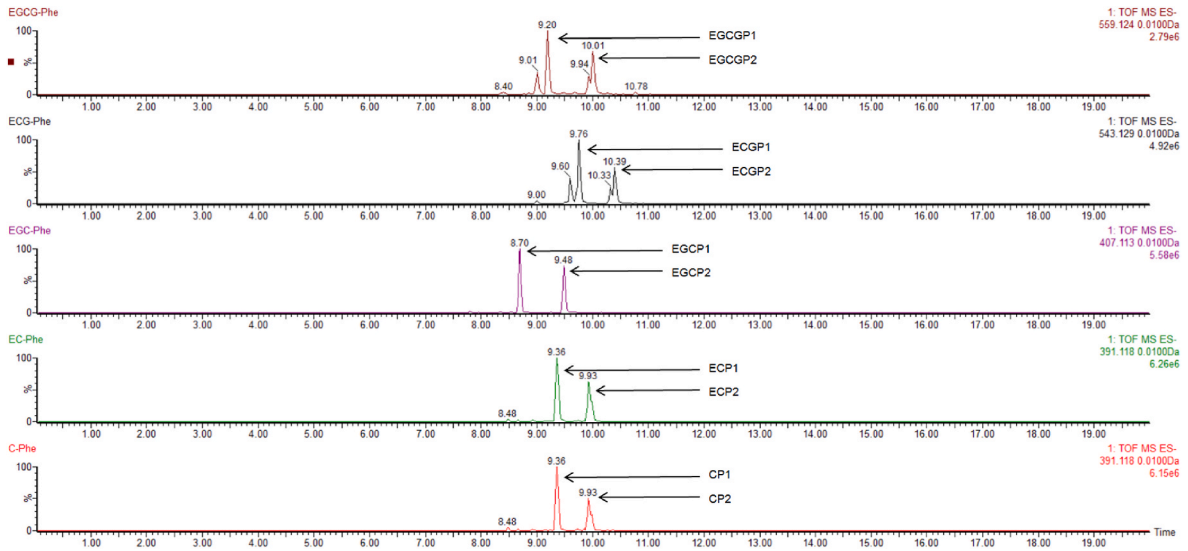

Fig. 6. Extracted ion chromatograms of phenylacetaldehyde-catechin adducts in diethylene glycol model system by MS.

previous studies which suggested that EGCG may inhibit PhIP formation mainly via trapping phenylacetaldehyde, instead of their free radical-scavenging abilities (Zhu et al., 2016; Cheng et al., 2009). In addition, the ability of ECG, EGC and EC inhibiting PhIP in PhIP chemical model system was also significantly positive correlated with their phenylacetaldehyde-trapping ability at the 0.05 level (bilateral). In the rest of the case the two abilities were significant positive correlated at the 0.01 level (bilateral) for C.

To sum up, the roles of catechins in Maillard reaction related to HAAs formation maybe not only just being free radical-scavenging agents, but also maybe phenylacetaldehyde-trapping.

3.9. Analysis the phenylacetaldehyde-catechin adducts in diethylene glycol chemical model system

Diethylene glycol chemical model system was different from the typical PhIP chemical model system, which could provide the medium for direct interaction between phenylacetaldehyde and catechins to solve the insolubility of phenylacetaldehyde in aqueous reaction system.

In this study, ten phenylacetaldehyde-catechin adducts were detected in the diethylene glycol chemical model reaction system by UPLC-MS/MS, and their extracted ion chromatograms was shown in Fig. 6. Similar studies had proved that the sites of phenylacetaldehyde adding to flavonoid were the C-6 or C-8 of ring A on myricetin, luteolin, apigenin, quercetin, myricetin, naringenin and EGCG (Zhu et al., 2016; Cheng et al., 2008, 2009; Zhou et al., 2018). Combining the MSMS fragment ions of these adducts in Table 3 with the previous report, these adducts in our experiments were deduced to be 8-C-(E-phenylethenyl)-EGCG (EGCGP1), 6-C-(E-phenylethenyl)-EGCG (EGCGP2), 8-C-(E-phenylethenyl)-ECG (ECGP1), 6-C-(E-phenylethenyl)-ECG (ECGP2), 8-C-(E-phenylethenyl)-EGC (EGCP1), 6-C-(E-phenylethenyl)-EGC (EGCP2), 8-C-(E-phenylethenyl)-EC (ECP1), 6-C-(E-phenylethenyl)-EC (ECP2), 8-C-(E-pheny lethenyl)-C (CP1), 6-C-(E-phenylethenyl)-C (CP2).

The existence of these adducts confirmed the hypothesis that catechins exhibit multiple mechanisms of action complementary to the traditional view predominantly as antioxidants. In recent years, emerging evidence suggests that flavonoid were effective scavenging agents of reactive carbonyl specie (RCS) in foods other than phenylacetaldehyde (Zhu et al., 2016; Cheng et al., 2009). So far, besides phenylacetaldehyde, a wide spectrum of RCSs is reported to be scavenged by natural polyphenol compounds, such as formaldehyde, acetaldehyde, glyoxal, methylglyoxal and acrolein (Zhu et al., 2009; Zamora et al., 2014).

3.10. Structure-activity relationship of catechins on HAAs inhibition and inhibitory mechanism

Currently, limited studies have been carried out to determine the structure–activity relationship between the chemical structure and the HAAs inhibitory activity of catechins. Studies (Salazar et al., 2014) have shown that the number and position of phenolic hydroxyl groups of polyphenols affect the inhibitory activity of HAAs. Our results speculated that the introduction of hydroxyl group to the ring B may increase the inhibitory effects, whereas the galloyl group in the ring C of catechins may play an important role in inhibiting the formation of PhIP and MeIQx. EGCG and ECG have one more esterified gallic acid at the C-3 position in the ring C than EGC, EC and C, and EGCG got one more hydroxyl group in the ring B than ECG, which made EGCG have the best inhibiting ability and ECG took the second place. Then, EGC was structurally similar to EC and C, with an additional hydroxyl group adjacent to the *o*-diphenolic structure in the ring B enhancing the antioxidant activity, which make EGC have the third inhibiting ability. EC and C neither have esterified gallic acid at the C-3 position in the ring C nor the additional hydroxyl group adjacent to the *o*-diphenolic structure in the ring B, which make them the two weakest inhibitors of the five catechins. The inhibitory effects of polyphenols on HAAs formation could be attributed to the combined effects of different structural parameters. However, further studies are still needed to clarify how these

Table 3
Characterization of phenylacetaldehyde-catechin adducts in diethylene glycol model system.

Adducts	Formula	m/z [M-H]$^-$	Retention time/min	Major and important MS^2 ions	Identification
EGCGP1&EGCGP2	$C_{30}H_{24}O_{11}$	561.1	9.20, 10.01	169.0, 227.1	8-C-(E-phenylethenyl)EGCG or 6-C-(E-phenylethenyl)EGCG
ECGP1&ECGP2	$C_{30}H_{24}O_{10}$	545.1	9.76, 10.39	169.0, 227.1, 289.1, 391.1	8-C-(E-phenylethenyl)ECG or 6-C-(E-phenylethenyl)ECG
EGCP1&EGCP2	$C_{23}H_{20}O_7$	407.1	8.70, 9.48	165.0, 177.0, 239.1	8-C-(E-phenylethenyl)EGC or 6-C-(E-phenylethenyl)EGC
ECP1&ECP2	$C_{23}H_{20}O_6$	391.1	9.36, 9.93	205.1, 239.1, 289.1, 301.1, 161.1	8-C-(E-phenylethenyl)EC or 6-C-(E-phenylethenyl)EC
CP1&CP2	$C_{23}H_{20}O_6$	391.1	9.36, 9.93	205.1, 239.1, 289.1, 301.1, 161.1	8-C-(E-phenylethenyl)C or 6-C-(E-phenylethenyl)C

chemical groups of catechins work in the inhibition of HAAs.

4. Conclusion

The inhibitory potency of five catechins against PhIP and MeIQx in chemical models followed the order: EGCG > ECG > EGC > C > EC. The inhibitory effects of EGCG and ECG on the formation of PhIP and MeIQx were significantly superior, particularly in the case of EGCG. In terms of structure of catechins, the introduction of hydroxyl group to the ring B, and galloyl group in the ring C of catechins both could increase the inhibitory effects of HAAs. As for the inhibitory mechanisms of catechins on HAAs, the inhibitory effects of ECG, EGC, EC, and C were found to be more closely associated with their free radical-scavenging ability. In contrast, the phenylacetaldehyde-trapping ability may represent a more significant mechanism by which EGCG inhibits PhIP in a chemical model system. In the future, the conclusion obtained in this study will be verified in the heat-treated meat products. Deeper studies are needed to identify the trapping ability of catechins in more kinds of RCS and clarify the specific chemical groups of catechins work in the inhibition of HAAs formation.

CRediT authorship contribution statement

Ruiwei Xie: Methodology, Investigation, Data curation, Writing – original draft. **Haolin Zhang:** Methodology, Investigation, revision of original draft. **Xiaomei Lv:** Investigation. **Qiuyi Lin:** Investigation. **Bing-Huei Chen:** Supervision, Conceptualization. **Yu-Wen Lai:** Supervision, Conceptualization. **Lei Chen:** Investigation. **Hui Teng:** Investigation. **Hui Cao:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

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

No data was used for the research described in the article.

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