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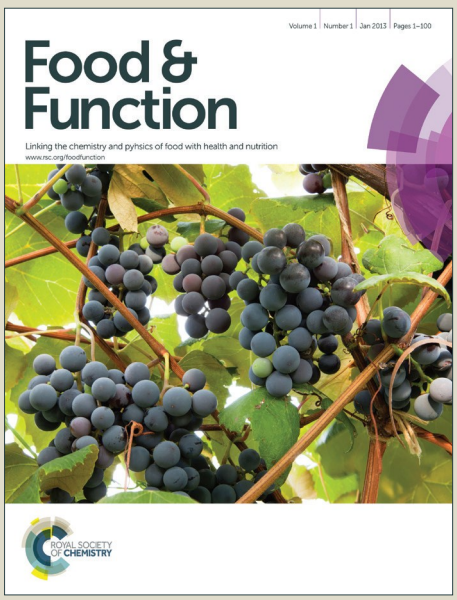

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Inhibitory effects of selected dietary flavonoids on the formation of total heterocyclic amines and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) in roast beef patties and in chemical models

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Abstract

In this study, the inhibitory effects of eight kinds of dietary flavonoids on the formation of heterocyclic amines (HAs) were investigated in roast beef patties. The results showed that most of them exhibited significant inhibition on both total HAs and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) as one of the most abundant HAs. Among them, phlorizin, epigallocatechin gallate (EGCG), and qucercetin were found to be the most effective in both the reduction of total HAs (55~70%) and PhIP (60~80%). The reaction activities between flavonoids and phenylacetaldehyde (key intermediate of PhIP formation) showed a good correlation with the inhibition of flavonoids PhIP formation in aqueous model system (R^2 = 0.8904) and di(ethylene) glycol reaction system (R^2 = 0.6514). However, no significant correlation was found between the antioxidant capacities of flavonoid and PhIP formation (R^2 = 0.2359). The postulated adducts of flavonoids-phenylacetaldehyde were further confirmed by LC-MS analysis in chemical models. Since phenylacetaldehyde was the chief intermediate of PhIP formation, these results combined suggested that the inhibitory effects of flavonoids on PhIP formation were mainly depended on their abilities to trap phenylacetaldehyde, instead of their antioxidant capacities.

Keywords: heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), dietary flavonoids, phenylacetaldehyde, phenylacetaldehyde-flavonoid adducts

Introduction

The treatment of heating on meat and fish products generates a class of heterocyclic amines (HAs).[1,2] These HAs are mainly formed with amino acids, sugars and creatin(in)e or other aldehydes as precursors during food processing.[3] Although they present at very low concentration (at ppb range) in foods, the safety issues regarding their mutagenicity have been known in bacteria mutagenicity[4] and in animal carcinogenicity.[5] Their potential health risks for human beings have been further recognized by people during the recent years. Many evidences have proved that the intake of meat rich in HAs is associated with an increased risk for several kinds of cancer, such as pancreatic cancer,[6] colon cancer,[7] prostate cancer,[8] breast cancer,[9] and lung cancer.[10] Up to now, more than 25 HAs have been identified from the food samples with structures elucidation, and 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP) is one of the most abundant HAs in addition to 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx).[11,12] Because the precursors of HAs are food components and HAs are hard to are hard to be removed once they are already formed, it is significant to find out potent inhibitors in their formation pathways to reduce their amount in foods.

The identification of possible inhibitors from dietary plants is one of the effective strategies for their easy incorporation in daily cuisine. Plant extracts, including fruit extracts, spices, and essential oils are regarded as health-promoting foods with anti-oxidant and anti-tumor effects.[13,14] They were also reported to have the ability to effectively inhibit the formation of HAs.[3,15-20] Certain dietary phenolic compounds from the plant extracts

were also identified as powerful inhibitors.[18-20]

The popular mechanism involved in the inhibition of HA formation by these food-derived phenolic compounds is proposed as free radical scavenging (antioxidant) activity in previous studies[21]. Recently, another possible mechanism was proposed that these substance may inhibit HAs formation through trapping the precursors or intermediates in HA formation pathways.[18,19,22] The postulated inhibition mechanism of dietary flavonoids (naringenin and EGCG) on PhIP formation was the direct reaction with the intermediate phenylacetaldehyde. However, there has been debate on the HA inhibition mechanism because of complexity of the reaction matrix, and it is significant to clarify the principal mechanism responsible for dietary flavonoids' intervention on HA formation.

The aims of present study were to evaluate the inhibitory effects of dietary phenolic compounds on HA formation; to investigate the inhibitory mechanism of total HAs and PhIP formation by different structure types of flavonoids (Figure 1); and find out the correlation of possible mechanism involved. Effects of flavonoids on total HA formation is firstly evaluated in roast beef patties UPLC/MS analysis. For the mechanism elucidation, the intermediates-scavenging ability was investigated in chemical models and PhIP's precursor phenylacetaldehyde was chosen as the typical intermediates in HA formation with further confirmation of these adducts in roast beef patties. The antioxidant capacity of these flavonoids was accessed by ABTS radical scavenging assay. Finally, the relationship between the two mechanism in HA formation was calculated in order to clarify their contribution.

Materials and methods

Chemicals

Phenylacetaldehyde, diethylene glycol, ethyl acetate, methanol and acetonitrile were purchased from Sinopharm Chemical Reagent Co, Ltd. (PR China). Apigenin, luteolin, kaempferol, quercetin, genistein, phlorizin, and epigallocatechin gallate (EGCG) were purchased from Nanjing Ze Lang Medical Technology CO, Ltd. (PR China). The standards of HAs 2-amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP), 2-amin-1, 6-dimethylimidazo[4,5-b]pyridine (DMIP), 2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo [4,5-f]-quinoxaline4,8-(DiMeIQx), 1-methyl-9H-pyrido [3,4-b]indole (Harman), 9H-pyrido[3,4-b]indole (Norharman), and 2-amino-1,5,6-trimethylimidzao[4,5-b]-pyridine (1,5,6-TMIP) were purchased from Toronto Research Chemicals (Toronto, Canada). Oasis MCX cartridges (3 mL/60 mg) were purchased from Waters (Milford, Massachusetts, USA). 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and naringenin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Caffeine was provided by J&K Chemical Ltd. (Shanghai, China). Fresh ground beef (hindquarter) was purchased from local supermarket.

LC/MS analysis of HAs in roast beef patties

The ground beef (40 g) was thoroughly mixed with different kinds of powdered flavonoids (0.2 mM), and then was formed into beef patties with a Petri dish (6 × 1.5 cm). The patties were covered by a Petri dish and were allowed to incubate under 4 ℃ conditions for 8 h in the refrigerator before frying. The beef patties were roasted in an electric oven for 20 min

(10 min per side) at 230 ℃. Three samples were taken for each treatment in each experiment.

Beef patties were homogenized after cooling according to the previous method.[23] An amount of 5 g roast beef was dissolved into 30 mL 1 M NaOH and homogenized for 2 min. After that, the viscous solution was mixed with diatomaceous earth and poured into empty extrelut columns with ethyl acetate as the eluent. The concentrated eluent was then transferred into the Oasis MCX cartridges. The cartridges were washed with 3 mL 0.1 M HCl and 3 mL MeOH twice, respectively. The analytes were eluted with 2 mL of MeOH-aqueous ammonia mixture (19:1) and concentrated under a nitrogen flushing and the residue was dissolved in 300 μL MeOH and subject to UPLC/MS analysis.[20,23-25]

Fresh working standard solutions were prepared daily by mixing and diluting each stock standard solution in methanol to obtain different concentrations with similar peak areas. Caffeine (CAF) was used as the internal standard (IS).

HAs were analyzed on a Waters UPLC with a tandem mass spectrometry. Separation was carried out on an Acquity BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm), with 10 mM ammonium acid (solvent A) and acetonitrile (solvent B) as a mobile phase. The total flow was 0.3 mL/min, with a following gradient elution: 0 min 90% A: 10% B; 0.1 min 90% A: 10% B; 18 min 70%A: 30% B; 20 min 100% B; 20.1 min 90% A: 10% B, total flow was 0.3 mL/min. The injection volume was 1 μL. MS spectrometry conditions were as follows: positive ion mode, spray voltage 2.8 V, ion source temperature 120 ℃, desolvation temperature 350 ℃.

Evaluation of phenylacetaldehyde-scavenging capabilities in aqueous model system by GC-MS

The model reaction system was carried on according to the method of previous study[20] with some minor modifications. The mixtures of 0.4 mM phenylalanine, 0.2 mM glucose, 0.1 mM creatinine, and 0.4 mM flavonoids were dissolved in the mixed solution containing 15 mL phosphate buffer (0.1 M, pH 7.0) and 5 mL di(ethylene) glycol. The reaction mixtures were then transferred into crew cap Tuf-Bond Teflon fitted glass reaction vials (40 mL capacity) and heated in an oil bath at 130 ℃ for 30 min. The reaction mixtures were subsequently cooled under ice bath for 40 min and then extracted by 20 mL ethyl acetate two times. The ethyl acetate extract was collected from the two-phase solution and diluted with ethyl acetate to 100 mL, dried with anhydrous sodium sulfate, filtered, and then subjected to GC-MS analysis.

The samples were analyzed on a Shimadzu gas chromatographe (GC-2010 Plus) equipped with an autosampler (AOC-20i). Separation was carried out on a capillary column of PEG20M. Analyses methods were as follows: inlet temperature, 50 ℃; temperature program, 10 ℃/min; up to 220 ℃ and hold for 2 min; column gas flow, 3mL/min (N_2); injection volume, 0.8 μL. The correlation coefficient (r^2) for the phenylacetaldehyde standard curve was 0.9882.

Direct reaction between flavonoids and phenylacetaldehyde in di(ethylene) glycol model system

This reaction was according to the previous study[20] with some minor modifications. Firstly,

putting 140 μL phenylacetaldehyde and 0.2 mM flavonoids into a two-phase solution which contained 21.5 mL di(ethylene) glycol and 3.6 mL Milli-Q water. Then the solution was mixed by a vortex and heated at 130 ℃ for 2 hrs. The reaction mixtures were subsequently cooled by an ice-water bath for 30 min. After cooling, the reaction solution was poured into a separation funnel, 20 mL Milli-Q water was added and mixed, and then the mixed solution was extracted by 40 mL ethyl acetate two times. The ethyl acetate extract was collected from the two-phase solution and diluted with ethyl acetate to 100 mL, dried with anhydrous sodium sulfate, filtered, and then subjected to GC-MS analysis for the residual phenylacetaldehyde.

The formation of phenylacetaldehyde-flavonoid adducts was detected by a Waters UPLC-Q-TOF system, equipped with a SYNAPT mass spectrometer and a Waters Acquity UPLC. Separation was carried out on an Acquity BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm). The mobile phase composed of aqueous solution containing 0.1% formic acid (solvent A) and methanol (solvent B). Gradient elution was as follows: 0 min, 60% A: 40% B; 5 min, 0% A: 100% B; 7 min, 0%A: 100% B; 7.10 min, 60% A: 40% B; 12 min, 60% A: 40% B, a flow rate of 0.3 mL/min. In order to separate phenylacetaldehyde-quercetin adducts, an adjusted gradient elution was used as follows: 0 min, 95% A: 5% B; 1 min, 95% A: 5% B; 7 min, 70% A: 30% B; 9 min, 40% A: 60% B; 15 min, 0% A: 100% B; 17 min, 0% A: 100% B; 17.1 min, 95% A: 5% B; 20 min, 95% A: 5% B. The MS spectrometry conditions were as follows: negative ion mode, spray voltage 2.8 V, cone voltage 31 V, source temperature 110 ℃, desolvation temperature 400 ℃, scan range 100-1500 Da.

148 **Antioxidant capacity of flavonoids**

149 This assay was performed according to the previous method[26] with some minor
150 modifications. Briefly, 7 mM $ABTS^+$ salt solution was first reacted with potassium
151 peroxosulphate solution (final concentration: 2.45 mM). The reaction mixture was allowed
152 to keep in the dark for 16 h at room temperature. The resultant $ABTS^+$ solution was then
153 diluted with deionized water to an UV absorbance of 0.70 ± 0.02 at 734 nm before use. All
154 the test flavonoids were predissolved in DMSO. Each sample solution (final concentration:
155 0.05 mM) or standard (different concentrations of Trolox) was mixed with diluted $ABTS^+$
156 solution and absorbance was taken at 734 nm on a UV-5300PC Spectro-photometer
157 (Metash Instrument Co., Ltd, Shanghai, China) after 6-min incubation. Results were
158 expressed as TEAC values (mM Trolox/mM flavonoid compound) with triplicate analyses.

159 **Extraction and detection of adducts in roast beef patties**

160 Roast beef (20 g) was mixed with MilliQ water (50 mL) and homogenized for 5 min. After
161 that, the cloudy suspensions were extracted with ethyl acetate (200 mL) for two times. The
162 concentrated elute was then evaporated to dryness and defatted with dichloromethane.
163 Finally, the dryness was dissolved in methanol (5 mL) for UPLC/MS analysis.

164 **Statistical analysis**

165 Statistical analysis was performed according to our previous study.[25] MassLynx 4.1 SCN
166 805 software was used to carry out the data acquisition. External calibration curves,
167 obtained by linear regression of a plot of the standard/IS peak area HAs ratios against the
168 HA concentrations, were used to calculate the compound concentrations in the samples. In

a spiked test, the original HA content in roasted pork was subtracted to calculate the recoveries. The statistical significance was compared between the control and experimental groups by one way analysis of variance (ANOVA) followed by an appropriate post hoc test (Tukey's multiple comparison test) using Statistics 9.0 to make the comparison, and post-hoc comparison was carried out using Tukey tests with a confidence level of 95%. Statistical significance is defined as $P < 0.05$.

Results and discussion

Inhibition effects of flavonoids on HAs and PhIP formation in roast beef patties

Dietary flavonoids present in fruits, vegetables, spices, and beverages, and most of them are taken as food ingredients and play an important role on human health. In this study, eight flavonoids with different structural types (Figure 1) were evaluated for their effects on the formation of HAs in roast beef patties. UPLC/MS analysis was used to detect the content of seven main HAs in roast beef patties, they were PhIP, DMIP, MeIQx, 4,8-DiMeIQx, Harman, Norharman, 1,5,6-TMIP, and the total content of HAs was the sum of these seven HAs. Harman, Norharman, 4,8-DiMeIQx, MeIQx, DMIP, 1,5,6-TMIP, and PhIP were identified with corresponding yields of 1.04 ± 0.07, 3.74 ± 0.15, 1.42 ± 0.16, 1.93 ± 0.06, 4.24 ± 0.08, 1.35 ± 0.23, and 10.73 ± 1.02 ng/g, respectively. Among all of seven HAs, the amount of PhIP was highest, and this is in agreement with previous reports.[18,19] As shown in Table 1, most of these eight kinds of flavonoids at 0.2 mM could significantly ($P < 0.05$) inhibit the formation of these seven HAs, except for apigenin on MeIQx formation, EGCG on 1,5,6-TMIP formation, and luteolin on PhIP formation. From

Table 1 and Figure 1, certain structure-activity relationship might be implied. Among all of eight flavonoids, phlorizin and EGCG displayed the strongest inhibition effects on the formation of total HAs and PhIP, with the inhibition rate up to 63.76% and 60.08%, 78.56% and 77.45% (Figure 2A), respectively, suggesting that chalcone and flavanol derivatives might be the most potential candidates as inhibition agents for HAs formation. Fruits, vegetables, spices, and beverages rich in these types of derivatives could be selected for further investigation. Among the four flavone derivatives, quercetin showed the strongest inhibition effects on the formation of total HAs and PhIP, reducing the level of total HAs and PhIP formed by 53.74% and 67.29%. After carefully examination on their structures, we found that the presence of a hydroxyl group at different positions, especially the B-ring and C-ring of the flavone skeleton, significantly affected their inhibition effects on HAs and PhIP formation. A hydroxyl group presented at 3-position of C-ring much improved the inhibition effects on HAs and PhIP formation (quercetin *vs.* luteolin, kaempferol *vs.* apigenin). Simultaneous occurrence of a hydroxyl group at 3'-position of B-ring and 3-position of C-ring would greatly enhance the resultant inhibition effects on HAs and PhIP formation, such as luteolin, kaempferol, and quercetin.

Phenylacetaldehyde-scavenging effects of flavonoids in chemical models

Previous studies of HA formation have showed that the thermic HAs are generated from the reaction of free amino acids, creatin(in)e, and hexoses. These precursors undergo further dehydration and cyclization to form the pyrrole, pyridine and pyrazine derivatives, and such heterocyclic pyrroles, pyridines and pyrazines will undergo further transformation

with participation of Strecker aldehydes and creatin(in)e to produce different HAs.[27-29] This implies that the scavenging or trapping of these aldehydes can be a possible strategy to reduce HAs during heating. In order to further explore the inhibition mechanism of these flavonoids on the formation of HAs and investigate whether they have similar inhibition mechanism or not, PhIP, the most abundant HA in most meat samples, was taken as the research focus.

PhIP, comprising about 70% of the daily intake of HAs in the United States,[30] is formed from the Maillard reaction among phenylalanine, glucose and creatinine.[28] The aqueous chemical model employed in this study is a typically PhIP-producing model. In this model, phenylacetaldehyde was transformed from phenylalanine and was identified by GC-MS as the chief volatile compound and lead to the formation of PhIP with creatinine.[20] Theoretically, the trapping of such intermediate could effectively inhibit the formation of PhIP, and in fact, it was reported that the concentration of phenylacetaldehyde was dose-dependent with the addition of phenylacetaldehyde-trapping agents.[19] Another chemical model employed in this study is di(ethylene) glycol reaction system, which provide the medium of direct interaction between phenylacetaldehyde and flavonoids because of the insolubility of phenylacetaldehyde in aqueous reaction system.

Cheng et al.[19,20] and Wong et al.[22] recently revealed that naringenin, EGCG, and vitamins (pyridoxamine) can strongly inhibit the PhIP formation by directly reacting with phenylacetaldehyde with adducts formation in model and real food systems. In our study, the trapping or scavenging of phenylacetaldehyde by eight flavonoids were evaluated in

both aqueous reaction system and di(ethylene) glycol reaction system. The results (Figure 2A) showed that all eight flavonoids could react with phenylacetaldehyde in aqueous PhIP-producing model system ($P < 0.05$). By GC-MS quantification of phenylacetaldehyde, it was found that phlorizin and EGCG still showed the strongest trapping capabilities of phenylacetaldehyde, and the trapping ratios were up to 85.10% and 88.81%, respectively. However, luteolin, genistein, and apigenin exhibited the relatively weaker trapping capabilities of phenylacetaldehyde, and the trapping ration ranging from 60.35% to 64.77%. In di(ethylene) glycol chemical reaction system which contains only phenylacetaldehyde and flavonoids, phlorizin, EGCG, and quercetin showed the best reaction capabilities with phenylacetaldehyde, with the ratios ranging from 50.01% to 52.73%. Nevertheless, luteolin and genistein displayed the weakest reaction capabilities, and the trapping ratios were 26.63 and 21.85% respectively (Figure 2B).

Relationship between phenylacetaldehyde-trapping and inhibition on PhIP formation

The relationship between the effects of flavonoids on PhIP formation in roast beef patties and the phenylacetaldehyde-scavenging capability of flavonoids in both aqueous reaction system and di(ethylene) glycol reaction system were analyzed by regression analysis. A good correlation ($R^2 = 0.8904$) was observed between the effects of flavonoids in roast beef patties on PhIP formation and the trapping capabilities of phenylacetaldehyde in aqueous reaction system (Figure 2C), and a strong correlation ($R^2 = 0.6514$) was also observed between the effects of flavonoids on PhIP formation and the trapping capabilities of phenylacetaldehyde in di(ethylene) glycol chemical reaction system (shown in Figure 2D).

These data indicated that the capacity of flavonoids in inhibition of PhIP formation was highly correlated with their trapping capabilities of phenylacetaldehyde.

Therefore, it is suggested that that the main pathway of flavonoids in inhibiting the formation of PhIP in roast beef patties is probably through trapping or reacting with phenylacetaldehyde, and this is in agreement with previous reports.[19,20] Furthermore, flavonoids might inhibit other types of HA formation via trapping various aldehydes as well. For instance, formaldehyde is the intermediate of IQ and MeIQx, whereas acetaldehyde is the intermediate of MeIQ and 4, 8-DiMeIQx.

Since the chemical models are simplified method for searching for HA inhibitors, and intermediate-trapping capability are related to the effects on HA formation in food model, investigation of the trapping capabilities of intermediate aldehydes in chemical models are recommended for the preliminary screening for HA inhibitors.

Analysis the phenylacetaldehyde-flavonoid adducts in roast beef patties and chemical reaction system

Several phenylacetaldehyde-flavonoid adducts, including [8-*C*-(*E*-phenylethenyl)-naringenin), 6-*C*-(*E*-phenylethenyl)naringenin, 8-*C*-(*E*-phenylethenyl)EGCG, 6-*C*-(*E*-phenylethenyl)EGCG] and pyridoxamine-phenylacetaldehyde adduct [(*E*-phenylethenyl)pyridoxamine] were isolated and identified from the model reaction by chromatographic methods, LC-MS and NMR spectroscopy in previous studies.[19,20,22] In this study, except for two phenylacetaldehyde-naringenin derivatives, 8-*C*-(*E*-phenylethenyl)naringenin) (**PN1**) and 6-*C*-(*E*-phenylethenyl)naringenin (**PN2**), other

seven phenylacetaldehyde-flavonoid adducts were isolated from the chemical reaction system by preparative HPLC and identified by LC-MS (Table 2), they were 8-*C*-(*E*-phenylethenyl)kaempferol (**PK1**), 6-*C*-(*E*-phenylethenyl)kaempferol (**PK2**), 8-*C*-(*E*-phenylethenyl)apigenin (**PA1**), 6-*C*-(*E*-phenylethenyl)apigenin (**PA2**), 8-*C*-(*E*-phenylethenyl)luteolin (**PL1**), 6-*C*-(*E*-phenylethenyl)luteolin (**PL2**), and 8-*C*-(*E*-phenylethenyl)qucercetin (**PQ**) (Figure 3).

Further confirmation of the phenylacetaldehyde-scavenging pathway was carried out by full scan model for the target analytes identification. Several phenylacetaldehyde-flavonoid adducts from roast beef were identified by LC-MS, with purified adducts as reference standards. It was found that beef samples treated with naringenin, kaempferol, apigenin, luteolin, and qucercetin contained phenylacetaldehyde-flavonoid adducts whose LC behavior and MS spectral characteristics completely matched with those of standard compounds (Figure 4). The proposed fragmentation pathways leading to the generation of characteristic fragment ions were listed in Table 2. Qucercetin-phenylacetaldehyde adducts were taken as the representative case to reveal the interactions between the precursor of PhIP and flavonoids. The total ion chromatograms (TIC) of the reaction mixtures that contain phenylacetaldehyde plus qucercetin showed distinct peaks with the molecular weight of 404 (m/z $[M - H]^-$ 403), assignable to an electrophilic substitution product between qucercetin and phenylacetaldehyde with the elimination of a water molecule. Collision-induced dissociation (CID) of the m/z 403 ion gave major products ion at m/z 253 and m/z 185, which was caused by Retro-Diels-Alder degradation of the C ring and

295 further loss of CO and CCO fragments. Such fragmentation pattern was found in both roast
296 beef patties and chemical models. The existence of these adducts confirmed the hypothesis
297 that flavonoid exhibit multiple mechanisms of action complementary to the traditional view
298 predominantly as antioxidants. In recent years, emerging evidence suggests that flavonoid
299 were effective scavenging agents of reactive carbonyl specie (RCS) in foods other than
300 phenylacetaldehyde. So far, a wide spectrum of RCS is reported to be scavenged by
301 phytochemicals, such as formaldehyde, acetaldehyde, glyoxal, methylglyoxal, acrolein and
302 4-hydroxy-*trans*-2-nonenal.[29,31,32] In this study, certain flavonoids worked as sacrificial
303 nucleophiles and consequently render the active sites of phenylacetaldehyde unavailable to
304 further reaction with creatinine that result in the formation of PhIP.

305 **Relationship between antioxidant capacity of flavonoids and their inhibition effects**
306 **on HA formation**

307 To investigate the contribution of dietary flavonoids' free radical-scavenging capability on
308 inhibition of formation on PhIP, the antioxidant activities of these flavonoids was evaluated.
309 One of the popular antioxidant assays was TEAC assay, which measures the antioxidant
310 activity of a substance compared to the standard Trolox. This method has been widely
311 applied to evaluate antioxidant activities of various components in foods, beverages and
312 nutritional supplements.[33] EGCG showed the strongest activity in this assay, and quercetin
313 takes the second place, while kaempferol was the least active one (Figure 5A). To
314 investigate the relationship between radical scavenging activity on PhIP formation, TEAC
315 values of eight flavonoids and their inhibitory effects of PhIP formation were analyzed by

regression analysis. However, a poor correlations (R^2 = 0.2359) were found between their antioxidant activities and the inhibition effects of PhIP formation (Figure 5B). The results in this study showed that antioxidant activities of flavonoids was not correlated with their inhibitory effects of PhIP formation, high antioxidant capacity does not necessarily lead to strong inhibitory effects on PhIP formation. Therefore, it is suggested that the capacity of flavonoids to inhibit PhIP formation was not correlated with their $ABTS^+$ radical scavenging abilities. It is in agreement with previous studies which also found the poor relation between antioxidant activities and inhibition of PhIP formation,[18] and in this study, the contribution of phenylacetaldehyde-scavenging was confirmed to be dominate instead of antioxidant activities in the inhibition of PhIP formation. The role of phenolic compounds in Maillard reaction related to HA formation should be more complex than just being free radical scavenging agents.

Conclusions

In conclusion, this study demonstrated that certain flavonoids could significantly inhibit the total HAs and PhIP formation in roast beef patties. Phlorizin and EGCG showed the strongest inhibitory capabilities on the formation of both total HAs and PhIP. Further study showed that all eight flavonoids could effectively reduce the content of key intermediate of PhIP formation, phenylacetaldehyde, in aqueous chemical reaction models and di(ethylene) glycol reaction system. A significant and strong correlation between the effects of flavonoids on PhIP formation and the trapping capabilities of phenylacetaldehyde were found in aqueous model system and in di(ethylene) glycol reaction system,

respectively. Combined with weak correlation between their antioxidant activities and the inhibition effects of PhIP formation, it is suggested that flavonoids may inhibit PhIP formation mainly via trapping its key intermediate, instead of their free radical scavenging abilities. The identification of phenylacetaldehyde-flavonoid adducts in roast beef patties further confirm the mechanism via scavenging of phenylacetaldehyde. Based on these observations, it is concluded that in foods during various processing procedures, scavenging of some critical intermediates such as RCS is important in intervention of formation of many harmful substances. Such as Strecker aldehydes formaldehyde is involved in the formation of IQ and MeIQx, whereas acetaldehyde is involved in the formation of MeIQ and 4, 8-DiMeIQx. As flavonoids are naturally-occurring with great abundance and widely diverse structural features, they could possess multiple functions besides traditional view as antioxidants. More work needs to be done to screen for more effective scavenging agents in inhibition of HA formation, and reduce the dietary intake of harmful HAs.

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Notes

The authors declare no competing financial interest.

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Figure captions

Figure 1. The structures of eight dietary flavonoids applied in this study.

Figure 2. (A) Relative percentages of the amount of total HAs and PhIP in roast beef system containing various flavonoids. (B) Relative percentages of the amount of phenylacetaldehyde in aqueous model reaction system and diethylene glycol-water model system containing various flavonoids. All treatments were made in triplicate. Bars with an asterisk indicate a significant difference from the control (Tukey, $P < 0.05$). (C) Correlation between the trapping capabilities of phenylacetaldehyde by flavonoids in aqueous model reaction system and the inhibition effects of flavonoids on PhIP formation in roast beef patties. (D) Correlation between reaction capabilities of flavonoids and phenylacetaldehyde in diethylene glycol reaction system and the inhibition effects of flavonoids on PhIP formation in roast beef patties.

Figure 3. Extracted ion chromatograms of adduct standards (A) and them in roast beef samples (B) (full scan model).

Figure 4. Structures of phenylacetaldehyde-flavonoid adducts obtained in this study

Figure 5. (A) Radical scavenging capacities of eight flavonoids determined by the TEAC assay. (B) Correlation between $ABTS^{+}$ radical scavenging and the inhibition effects on PhIP formation.

TABLES

Table 1. Effect of eight flavonoids on the formation of HAs in roast beef patties at 230 ℃ for 20 min

Treatments	HAs (ng/g)							
	Harman	Norharman	4,8-MeIQx	MeIQx	DMIP	1,5,6-TMIP	PhIP	Total HAs
Control	1.04 ±0.07^{a}	3.74 ±0.15^{a}	1.42 ±0.16^{a}	1.93 ±0.06^{a}	4.24 ±0.08^{a}	1.35 ±0.23^{a}	10.73 ±1.02^{a}	24.45 ±1.77^{a}
Apigenin	0.83 ±0.02^{b}	2.22 ±0.07^{b}	1.15 ±0.18^{b}	1.92 ±0.10^{a}	2.45 ±0.75^{b}	1.01 ±0.06^{b}	7.30 ±1.22^{b}	16.88 ±2.4^{b}
Luteolin	0.47 ±0.03^{c}	2.09 ±0.29^{c}	0.67 ±0.05^{c}	1.35 ±0.21^{b}	3.48 ±1.07^{c}	1.16 ±0.11^{c}	9.76 ±0.77^{c}	18.98 ±2.53^{c}
Kaempferol	0.43 ±0.03^{c}	1.84 ±0.07^{cd}	0.43 ±0.24^{d}	1.03 ±0.10^{c}	2.54 ±0.41^{d}	1.08 ±1.09^{d}	5.70 ±0.42^{d}	13.05 ±2.36^{d}
Quercetin	0.40 ±0.00^{c}	2.12 ±0.05^{ce}	0.79 ±0.14^{e}	1.52 ±0.04^{d}	2.47 ±0.24^{b}	0.48 ±0.12^{e}	3.53 ±0.20^{e}	11.31 ±0.79^{e}
Genistein	0.32 ±0.08^{d}	1.58 ±0.08^{f}	0.84 ±0.23^{f}	0.85 ±0.11^{e}	3.08 ±0.42^{e}	0.58 ±0.04^{f}	6.64 ±0.83^{f}	13.89 ±1.79^{f}
Naringenin	0.20 ±0.09^{e}	2.14 ±0.52^{ce}	0.03 ±0.04^{g}	0.39 ±0.18^{f}	2.29 ±0.73^{f}	0.85 ±0.12^{g}	4.87 ±0.49^{g}	10.77 ±2.17^{g}
Phlorizin	0.28 ±0.02^{d}	1.90 ±0.16^{g}	0.34 ±0.03^{h}	0.80 ±0.03^{g}	2.40 ±0.42^{g}	0.84 ±0.06^{g}	2.30 ±0.35^{h}	8.86 ±1.07^{h}
EGCG	0.38 ±0.02^{cf}	1.52 ±0.09^{h}	0.20 ±0.14^{i}	0.59 ±0.15^{h}	3.12 ±0.40^{h}	1.53 ±0.11^{h}	2.42 ±0.43^{i}	9.76 ±1.34^{i}

Values with superscripts in the same column are significantly different ($P < 0.05$); Data represent the mean ± SD of three treatment.

454 **Table 2.** Characterisation of adducts in roast beef patties and model systems

Adducts	Fornula	*m/z* $[M-H]^-$	Major and important MS^2 ions	Identification
PN1&PN2	$C_{23}H_{18}O_5$	373.1	279.1, 253.1, 209.1, 185.1, 119.1	8-*C*-(*E*-phenylethenyl)naringenin) or 6-*C*-(*E*-phenylethenyl)naringenin
PK1&PK2	$C_{23}H_{16}O_6$	386.9	310.2, 246.6, 264.9, 112.9	8-*C*-(*E*-phenylethenyl)kaempferol or 6-*C*-(*E*-phenylethenyl)kaempferol
PA1&PA2	$C_{23}H_{16}O_5$	370.9	112.9, 218.9, 238.8, 248.8, 296.9	8-*C*-(*E*-phenylethenyl)apigenin or 6-*C*-(*E*-phenylethenyl)apigenin
PL1&PL2	$C_{23}H_{16}O_6$	386.9	112.9, 248.8, 268.9, 284.9	8-*C*-(*E*-phenylethenyl)luteolin or 6-*C*-(*E*-phenylethenyl)luteolin
PQ	$C_{23}H_{16}O_7$	403.2	121.1, 185.1, 209.1, 253.1, 281.1	8-*C*-(*E*-phenylethenyl)qucercetin

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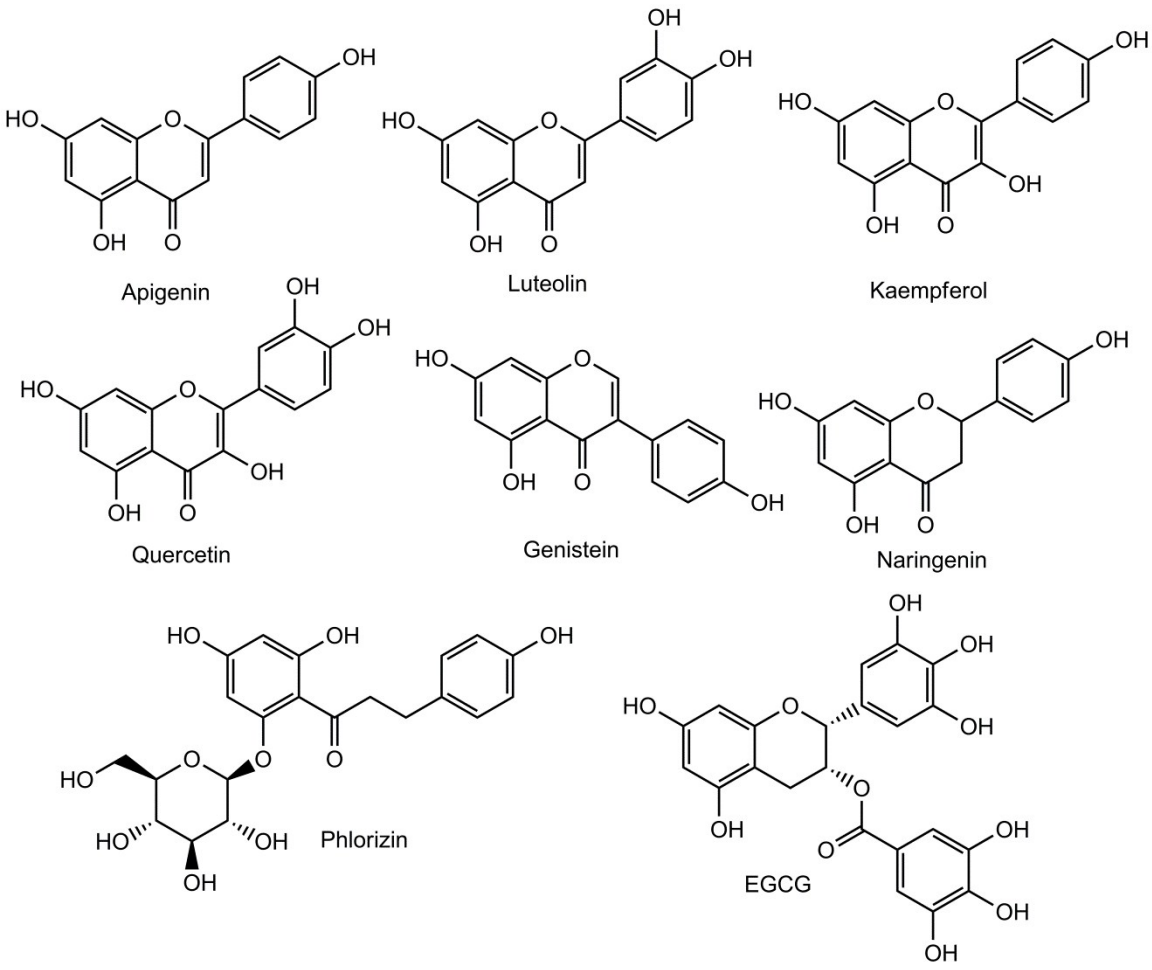


Figure 1

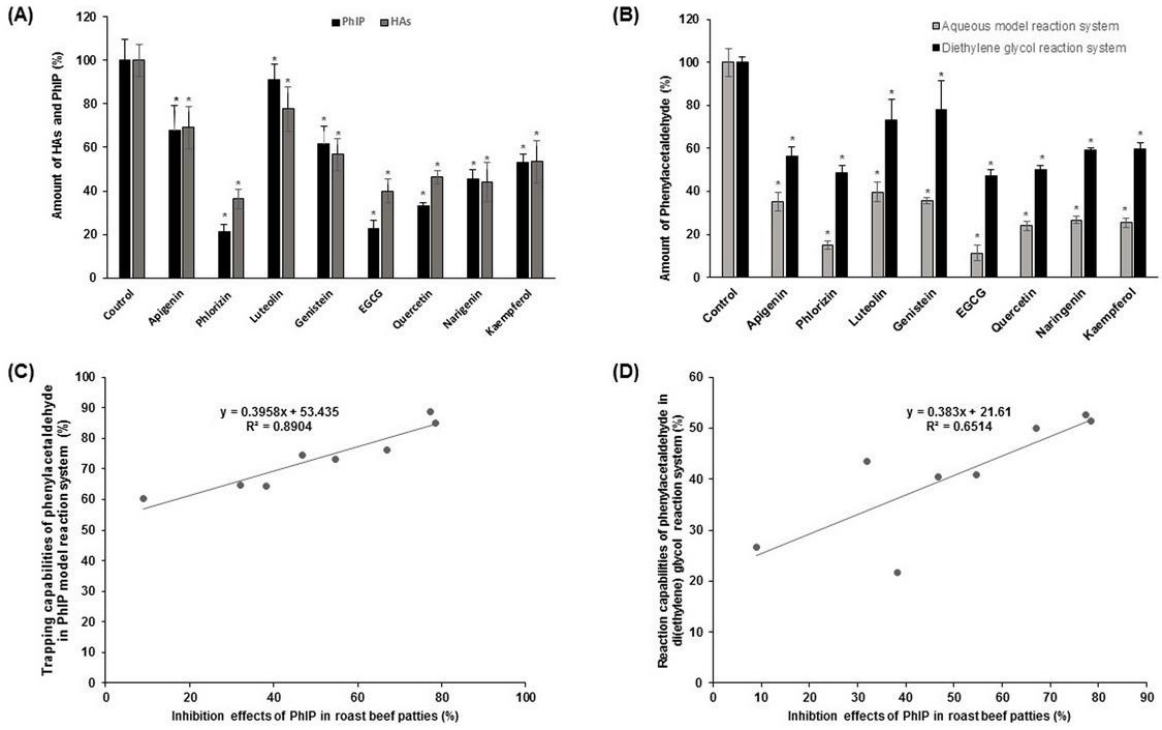
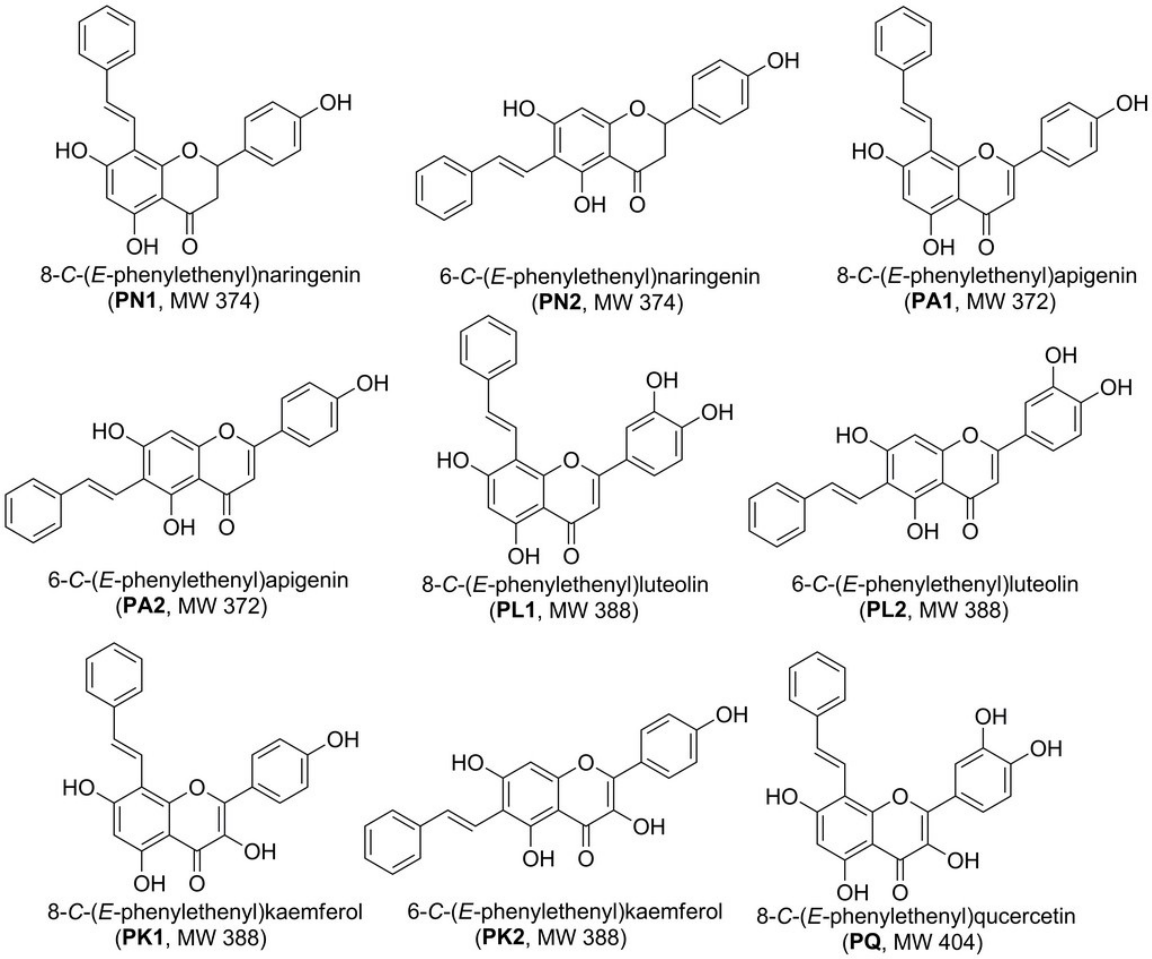


Figure 2



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474 **Figure 3**

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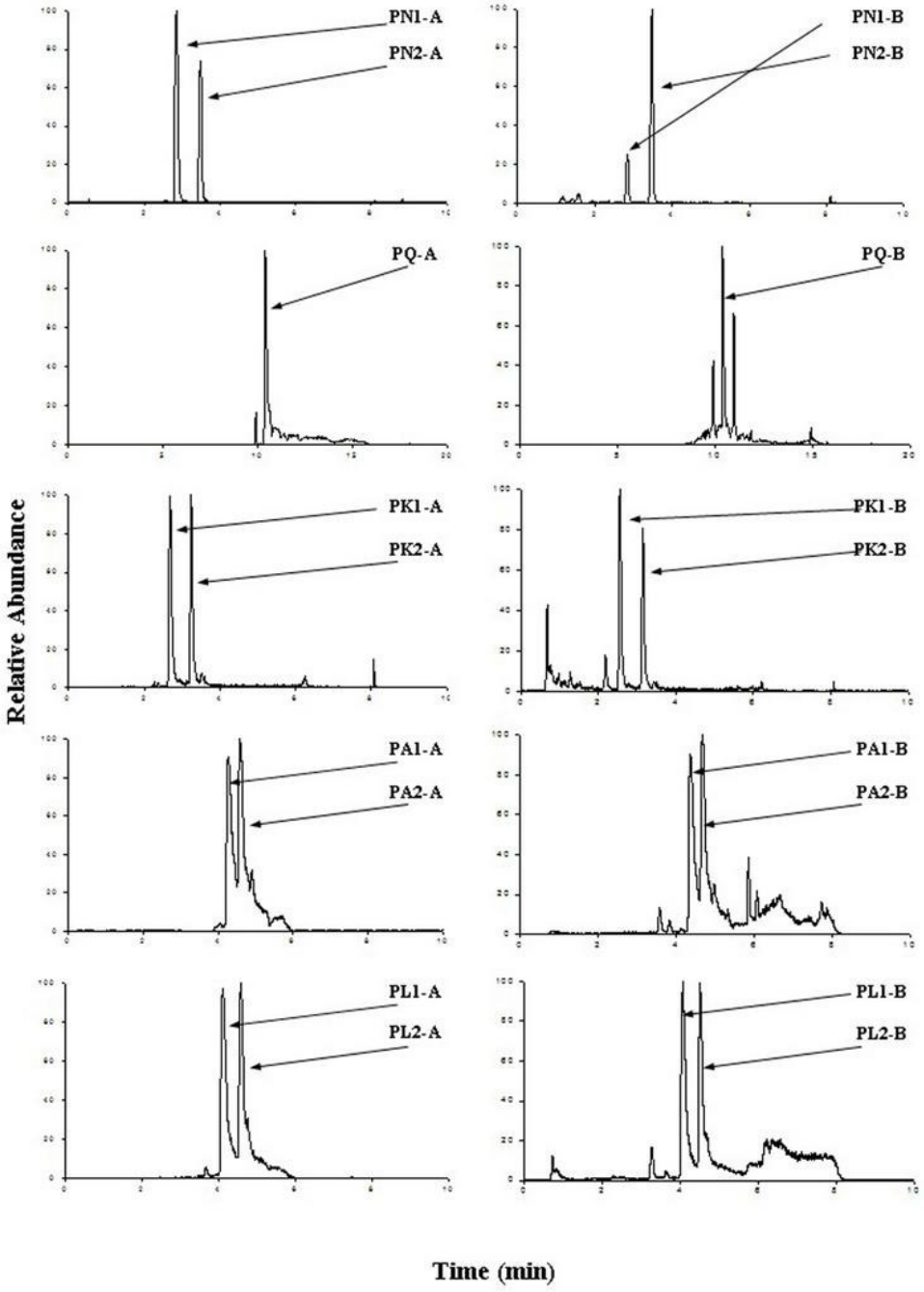

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477 **Figure 4**

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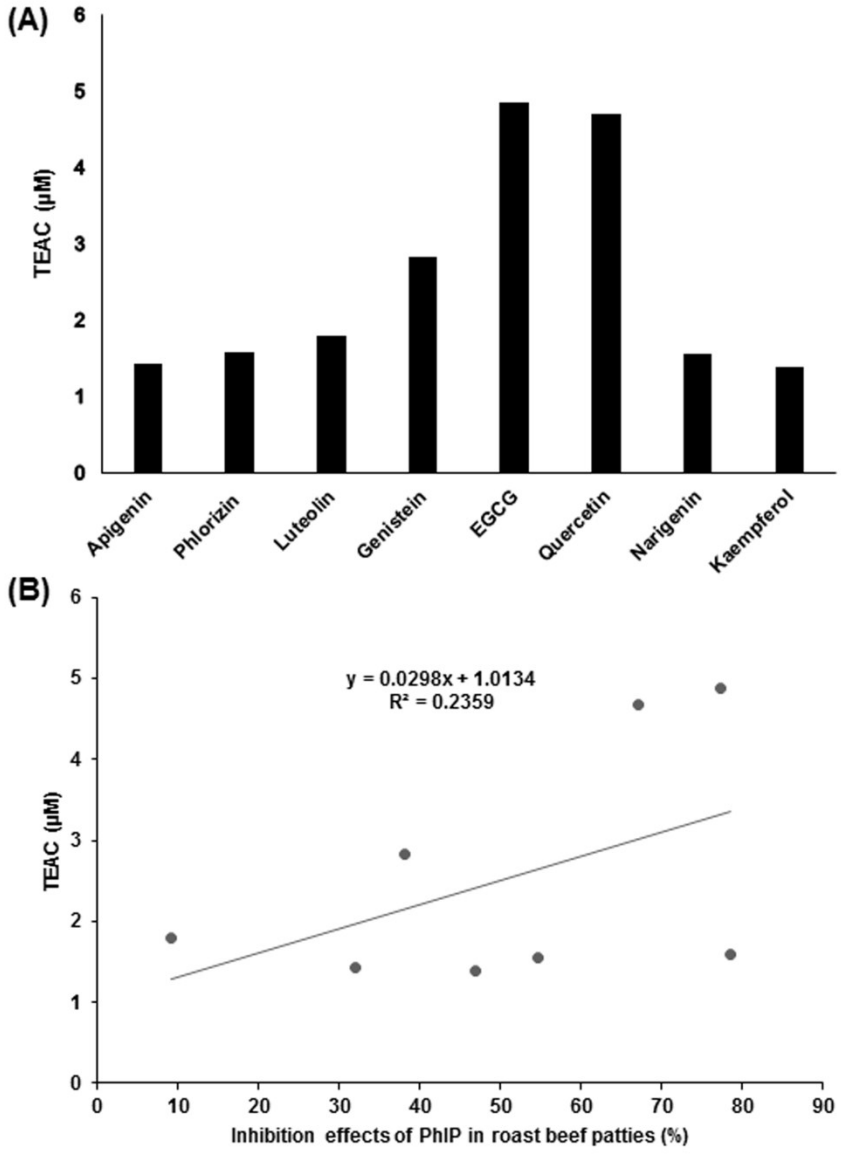

Figure 5

A table of contents entry

Dietary flavonoids effectively inhibit total HAs and PhIP formation in roast beef patties through scavenging of intermediates in formation pathways.