

The Inhibitory Effect of Spices and Flavonoid Compounds on Formation of 2-amino-1-methyl-6-Phenylimidazo [4,5-b] Pyridine (PhIP) in a Model System

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Abstract Heterocyclic amines (HCAs) are a class of mutagenic and carcinogenic compounds generated when muscle foods are cooked at high temperatures. Exposure to HCAs has been linked to human cancers, among them colon, prostate, breast, and pancreatic cancers. 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) is a common, potentially harmful HCA that forms via the Maillard reaction. The health consequences of consuming HCAs have caused the International Agency for Research on Cancer (IARC) to list PhIP as a “possible human carcinogen.” Spices and flavonoid compounds have received considerable attention for their beneficial effect against HCA formation in our daily foods. In this study, a model system with 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine in 90:10 diethylene glycol/water (v/v) was heat-treated at 180°C for 1 hour to test the formation of PhIP. Spices such as black pepper oil, piperine, D-limonene, P-cymene, and capsaicin and flavonoid compounds such as quercetin, apigenin, genistin, phlorizin, and catechin were added individually to the model system at three concentrations (125, 625, and 1250 ppm) to test their effect on PhIP formation. The PhIP contents were assessed using High-Performance Liquid Chromatography (HPLC). The results indicate that four out of five components of spices: black pepper oil, piperine, D-limonene, and capsaicin significantly ($p < 0.05$) reduced PhIP formation, while P-cymene had no significant effect on PhIP formation. All flavonoid compounds also had a significant ($p < 0.05$) effect on PhIP formation. These findings provide valuable information about spices and flavonoid compounds as protective agents against HCA formation.

Keywords: heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine, spices, flavonoid compounds, model system

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1. Introduction

Heterocyclic amines (HCAs) are a group of mutagenic and carcinogenic chemical compounds that are generated when muscle-rich foods are cooked at temperatures higher than 150°C [1]. The first discovery of carcinogenic compounds in heated meat was in 1939; Widmark, a Swedish chemist, applied extracts of fried horse meat to mice and found that they induced cancer [2]. HCA levels are detected in meat products depending on kind of meat product, cooking temperature, and cooking time [3]. Several epidemiological studies have reported that consuming fried meat products increases the risk of several kinds of cancers in humans [4,5]. Because of the health consequences of HCA, the International Agency for Research on Cancer (IARC) has listed

2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) as “possible human carcinogens,” and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) has been categorized as “a probable human carcinogen” [6]. These same HCAs have been classified as “reasonably anticipated to be a human carcinogen” by the National Toxicology Program of The U.S. Department of Health and Human Services [7]. PhIP has been reported to have more effect on DNA-cell damage than MeIQx and DiMeIQx [8]. It is also one of the most abundant HCAs produced in cooked meat and fish during normal cooking [9]. Therefore, researchers are interested in studying the effect of PhIP on foods eaten daily. Some studies of HCA use chemical model systems because it limits side reactions that occur in meats [1]. Model systems also allow researching the chemical interactions among the precursors and applied antioxidants

[10]. Meat and chemical model systems are used extensively to investigate the effect of antioxidants on HCA formation [11,12]. Many studies have suggested that antioxidants scavenge free radicals at various points along the HCA pathways, thus preventing HCA formation [13].

Black pepper is the dried, mature fruit of the *Piper nigrum* L. plant, belonging to the family Piperaceae and representing about 35% of the world trade in spices [14]. One of the important bioactive compounds of black pepper is piperine [15]. Black pepper has approximately 3.5% piperine, but levels increase based on pepper maturity and cultivar [14]. Previous studies have reported that piperine can be chemopreventive, antioxidants, immunomodulatory, anti-carcinogenic, and anti-inflammatory [16]. Oz and Kaya [17] studied piperine for its ability to inhibit HCA formation in a high-fat meatball, finding that PhIP levels decreased by 100% over control group. D-limonene and P-cymene are volatile compounds found in black pepper. Previous studies have reported that they have a beneficial health effect, working against mutagenic and carcinogenic formation [18]. Another type of pepper is hot red pepper (*Capsicum annum* L.). *Capsicum* belongs to the family of Solanaceae and goes by different names depending on location and pepper type [19]. Capsaicin has high amounts of vitamin C and total soluble phenolics [20] and is reported to reduce the risk of cancer [21]. In a study evaluating the effects of capsaicin (major compound in *Capsicum* spp) on mutagenic activity in roast beef patties, Zeng et al. [22] found that capsaicin inhibited PhIP by 80% to 98%. Flavonoids are a group of phenolic compounds widely found in nature with many biological functions; they are antioxidants, anti-inflammatory agents, and antimicrobial agents [23]. Despite the antioxidant properties of spices and flavonoid compounds, few studies have investigated whether these compounds inhibit HCA formation in chemical model systems. Therefore, the objective of this study was to investigate the effect of spices and flavonoid compounds in chemical model systems containing glucose, creatinine, and phenylalanine as the main precursors against PhIP formation.

2. Materials and Methods

2.1. Materials

Pure PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine) standard was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Spices (black pepper oil, piperine, D-limonene, P-cymene, and capsaicin) and flavonoids (quercetin, apigenin, genistin, phlorizin, and catechin) standards were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo., U.S.A.). D-glucose (99.5%), L-phenylalanine (98%), creatinine, diethylene glycol, and trimethylamine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., U.S.A.). Solvents and chemicals such as acetonitrile (high-performance liquid chromatography (HPLC) grade), methanol (HPLC grade), acetic acid, were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Deionized water was processed by a Sybron/Barnstead PCS unit (Barnstead/ThermoLyne, Inc.; Dubuque, Iowa, U.S.A.). 0.2 μ m syringe filters were provided by Fisher Scientific (Fair Lawn, N.J., U.S.A.).

2.2. Preparation of Model Systems

The effect of natural spices and flavonoid compounds on PhIP formation were evaluated using a model system with slight modifications [24]. The precursors, 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine were dissolved in 10% deionized water, 90% diethylene glycol (v/v) mixture and mixed by vortexing. The effect of the natural spices and flavonoids on PhIP formation were evaluated after individual addition (10 mg) of spices (black pepper oil, piperine, D-limonene, P-cymene, and capsaicin) and flavonoids (quercetin, apigenin, genistin, phlorizin, and catechin) to the model systems containing the precursors mentioned above. Samples without spices and flavonoids were used as control group.

The reactants were added to 1 mL reaction vial and then placed into brass vessels with 2 screw caps on the top/bottom, and 4 holes (1cm \times 1 cm) on the body of brass vessels to facilitate heat transfer from heating block to the reaction vials. Before heat treatment, all model systems contents were vortexed vigorously to make sure that all reactants were thoroughly mixed. The brass vessels were tightly closed and placed into a heating block (HP 5890; Agilent Technologies, Inc., Santa Clara, CA, USA). The heating temperature was set at 180°C for 1 hour, and the vessels were then immediately cooled down on ice for five min before further analysis. All model system samples were syringe filtered and diluted 1:10 with methanol before HPLC separation.

2.3. Analysis of PhIP

PhIP analysis was performed on HP 1050 series HPLC (Agilent Technologies) coupled with an HP 1050 series diode array UV-visible detector and an HP 1046 fluorescence detector. PhIP separation was performed using reversed-phase chromatography with a TSKgel ODS-80TM (4.6 mm \times 25 cm \times 5 μ m) column and a TSK guardgel ODS-80TM (3.2 mm \times 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan). The column temperature was set at 40°C. Each treatment (20 μ L) was injected onto a reverse-phase column and eluted with a mobile phase containing 0.01 M trimethylamine in deionized water (pH was adjusted to 3.6 with acetic acid) and acetonitrile at a flow rate of 1 ml/min. Mobile phase gradients were used following Puangsombat et al. [25] with slight modifications. A linear HPLC gradient started with 95% of 0.01 M trimethylamine and 5% acetonitrile and then decreased to 75% 0.01 M trimethylamine and 25% acetonitrile over 30 minutes. After 35 min, the initial ratio of 95% 0.01 M trimethylamine and 5% acetonitrile was maintained for 4 min to equilibrate the column. PhIP detecting was obtained by setting fluorescence detector at 437 nm emission and excitation of 229 nm.

2.4. Quantification and Statistical Analysis

A 250 ppm PhIP standard solution was prepared by dissolving 1mg of PhIP in 4 ml of methanol. A standard curve was prepared by analyzing PhIP standards at concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125,

0.015625, and 0.007813 ppm. The coefficient of determination (R^2) was 0.9976. Limit of detection (LOD) of PhIP was 0.201 ppm and the limit of quantification (LOQ) was 0.669 ppm. The results were analyzed using SAS version 9.4. Paired samples t-test was performed to determine significant differences between control group and the treatment groups. The significance level (P-value) was set at 0.05. All treatments were done in triplicate.

3. Results and Discussion

In this study, natural spices (black pepper oil, piperine, D-limonene, P-cymene, and capsaicin) and flavonoid compounds (quercetin, apigenin, genistin, phlorizin, and catechin) were investigated to determine their effect on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. Results showing the effect of natural spices and flavonoid compounds on PhIP formation (Table 1 - Table 2) are represented as means \pm standard error for each level of the treatment. All natural spices and flavonoid compounds were investigated individually at 3 levels: 125, 625, and 1250 ppm. Figure 1 shows PhIP eluted from the HPLC column at approximately 16.4 minutes.

3.1. Effects of Black and Red Pepper Compounds on PhIP Formation

Although some studies have evaluated the effect of black and red pepper compounds on PhIP formation in meat products, few studies have evaluated the effect of these spices on PhIP formation in chemical model systems. The first compound investigated in this study was black pepper oil (Table 1). On average, the reduction percentage of PhIP was 24%, 35%, and 34% for each respective level (125ppm, 625ppm, and 1250ppm). Black pepper oil at all three levels had a significant ($p < 0.05$) effect on PhIP formation. It appears that higher concentrations of black pepper oil reduced PhIP formation more than the lower concentration. Kelly and Smith [26] stated that when black pepper oil was added to chemical model system containing (glucose, creatinine, and phenylalanine) at different concentrations of 36 μ L, 71 μ L, 142 μ L, 285 μ L, and 550 μ L, PhIP levels were reduced by 31.4%, 30.8%, 25.7%, 23%, and 43.5% at each respective level. Black pepper has antioxidant and iron chelating abilities that lead to lower mutagenic activity [27]. Other studies reported that black pepper added to the surface of meat reduced PhIP levels by 100%, while the inhibitory effect of black pepper on other HCAs, including IQ, MeIQ, and 4, 8-DiMeIQx, ranged from 12 to 100% [17].

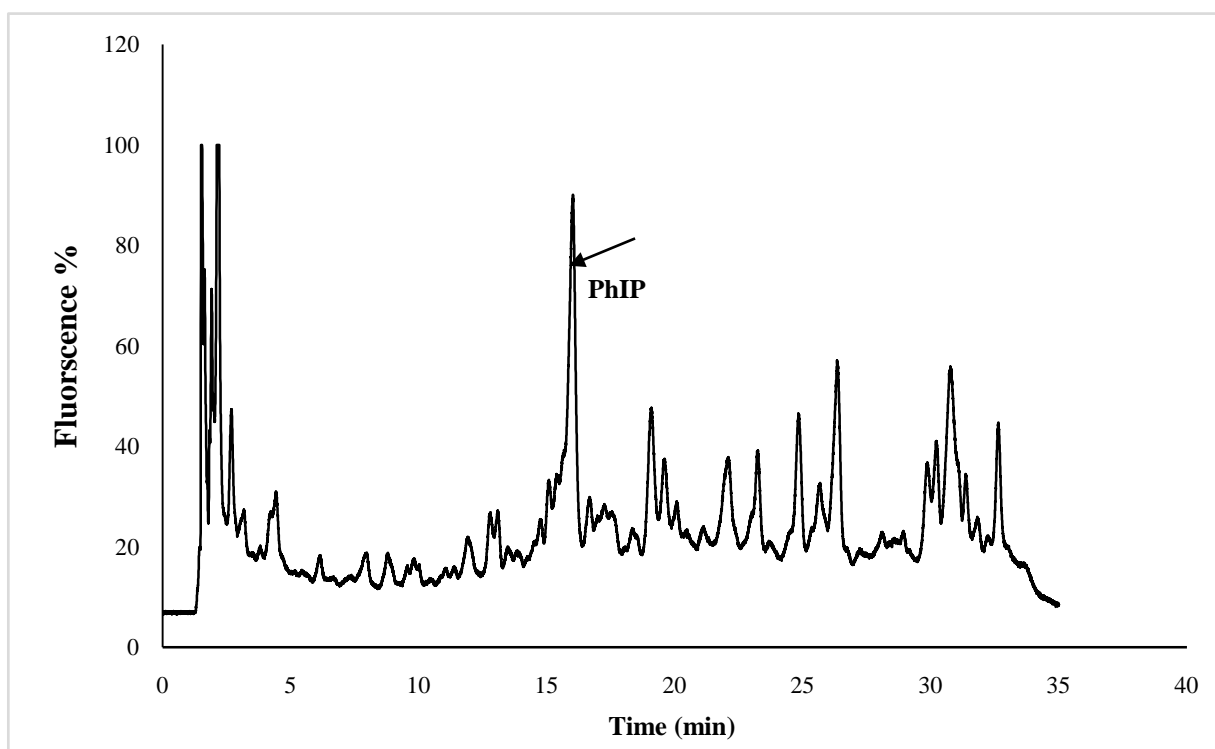


Figure 1. HPLC chromatograph of PhIP model system control (0.011 mmol glucose, 0.022 mmol creatinine, 0.022 mmol phenylalanine in 10% water, 90% diethylene glycol solution), separated using a TSKgel ODS-80TM (4.6 mm x 25 cm x 5 μ m) column and a TSKgel guardgel ODS-80TM (3.2 mm x 1.5 cm) guard column

Piperine was the second compound tested in the study (Table 1). On average, the percentage of PhIP was reduced by 15%, 15%, and 20% for each respective level (125ppm, 625ppm, and 1250ppm). Piperine added at all three levels significantly ($p < 0.05$) reduced PhIP formation. As shown in Table 1, the higher concentrations of piperine reduced PhIP formation more than lower concentrations. Kelly and Smith [26] reported similar

results, stating when piperine was added to model systems at different concentrations (4.02 mg, 8.04 mg, 16.14 mg, and 31.14 mg), PhIP content was reduced by 24%, 20%, 23.5%, and 29% at each respective level. Also, piperine inhibits PhIP formation in beef patties. Zeng et al. [22] evaluated the effect of piperine on PhIP formation in roast beef patties and found that adding 0.005%, 0.010%, and 0.015% of piperine to the surface of meat patties reduced

PhIP levels by 62%, 60%, and 56% at each respective level. Another compound evaluated in this study was D-limonene (Table 1). It is a chemical compound found in the peels of citrus fruits. On average, PhIP formation was reduced by 19%, 16%, and 15% for each respective level (125ppm, 625ppm, and 1250ppm). D-limonene at all three levels had a significant ($p < 0.05$) effect on PhIP formation. Table 1 shows the lower concentration of D-limonene reduced PhIP more than the higher concentration. P-cymene, an aromatic organic compound, was the fourth compound tested in the study (Table 1). On average, PhIP formation was reduced by 13%, 15%, and -7% for each respective level (125ppm, 625ppm, and 1250ppm). Adding P-cymene to the model system had no significant effect on PhIP formation. The data on P-cymene shows the higher concentrations of P-cymene enhanced PhIP formation. These results agree with previous research, where high concentrations of synthetic antioxidants enhanced HCA formation although low concentrations had some inhibitory effect [28,29]. Capsaicin, hot red pepper compound, was the final spices tested in this study (Table 1). On average, PhIP formation was reduced by 16%, 12%, and 12% for each respective level (125ppm, 625ppm, and 1250ppm). Capsaicin did have a significant ($p < 0.05$) effect on PhIP formation. Table 1 shows the lower concentration of capsaicin reduced PhIP more than the higher concentration. Adding capsaicin at the rate of 1% to the surface of meat before frying has been reported to reduce HCAs (IQ, MeIQ, 4,8-DiMeIQx, and PhIP) by 75 to 100% [17]. However, Zeng et al. [30] investigated the effect of 6 Chinese spices (pricklyash peel, star anise, fennel, cumin, capsaicin, and black pepper) on the HCA formation in roast beef patties. Their findings indicated that the only spice inhibiting HCA formation was prickly ash peel while the other spices enhanced HCA formation.

Table 1. Effect of black and red pepper compounds on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour

Black and red pepper compounds (ppm)		PhIP ($\mu\text{g/ml}$)*	Inhibition (%)
Black pepper oil	Control	1.84 \pm 0.083	
	125	1.40 \pm 0.083	24
	625	1.19 \pm 0.083	35
	1250	1.21 \pm 0.083	34
Piperine	Control	1.90 \pm 0.049	
	125	1.61 \pm 0.049	15
	625	1.62 \pm 0.049	15
	1250	1.53 \pm 0.049	20
D-limonene	Control	1.60 \pm 0.043	
	125	1.29 \pm 0.043	19
	625	1.34 \pm 0.043	16
	1250	1.36 \pm 0.043	15
P-cymene	Control	1.34 \pm 0.049	
	125	1.16 \pm 0.049	13
	625	1.14 \pm 0.049	15
	1250	1.43 \pm 0.049	-7
Capsaicin	Control	2.06 \pm 0.039	
	125	1.73 \pm 0.039	16
	625	1.81 \pm 0.039	12
	1250	1.81 \pm 0.039	12

Significant differences were observed between control and treatments at $p < 0.05$.

*Means \pm standard error for each level of the treatment (n=3).

3.2. Effects of Flavonoid Compounds on PhIP Formation

All flavonoid compounds reduced PhIP content to varying degrees compared to control group (Table 2). Also, all flavonoids tested in this study significantly ($p < 0.05$) reduced PhIP formation. Of all five flavonoids, genistin showed the highest inhibition effect on PhIP formation. On average, PhIP formation was reduced by 35% for the 125 ppm level, 42% for 625 ppm, and 29% for 1250 ppm. This indicates that genistin might be a powerful inhibitor against PhIP formation. Catechin ranked second. On average, PhIP formation was reduced by 22% for the 125 ppm level, 23% for 625 ppm, and 22% for 1250 ppm. Quercetin was third with, on average, PhIP formation was reduced by 15% for the 125 ppm, 15% for 625 ppm, and 30% for 1250 ppm. Apigenin, on average, PhIP formation was reduced by 25% for the 125 ppm level, 16% for 625 ppm, and 15% for 1250 ppm. Phlorizin was lowest, showing, on average, reducing PhIP formation by 14% for the 125 ppm level, 13% for 625 ppm, and 18% for 1250 ppm. The results of this study were consistent with previous research showing that these flavonoid compounds could effectively reduce PhIP formation in a chemical model system. In the literature, natural flavonoid compounds have been noted to protect against different types of cancer including lung, hepatocellular, and colorectal cancers [31,32,33]. Previous studies have also shown that flavonoid compounds protect against HCA formation. For instance, bamboo leaves (AOB) and flavonoids such as apigenin, luteolin, orientin, homoorientin, vitexin, isovitexin, isorhamnetin, fisetin, and hesperetin all inhibited PhIP formation in model systems [34]. Many studies have evaluated the effects of natural antioxidants on HCA formation in both chemical model systems and beef patties. Oguri et al. [29] investigated how luteolin, quercetin, catechins, Epigallocatechin Gallate (EGCG), and caffeic acid affected HCA formation in a chemical model system. The results showed reduced levels of PhIP and MeIQx by 3 to 75% compared to the control group. Previous studies have also reported that some flavonoids differ in how they inhibit PhIP formation than other types of HCAs. For instance, although quercetin inhibited PhIP formation the most, baicalein did not. In contrast, baicalein was more effective on MeIQx and 7, 8-diMeIQx formation than quercetin [35]. Cheng et al. [24] reported on three different phenolic compounds (proanthocyanidins, phloridzin, and chlorogenic acid), which when added to model systems to reduce PhIP and MeIQx formation showed that proanthocyanidins inhibited both PhIP and MeIQx the most, and phloridzin significantly ($p < 0.05$) inhibited PhIP formation. On the other hand, chlorogenic acid was a potent inhibitor of MeIQx but enhanced PhIP formation. Zhu et al. [36] studied the effect of eight different flavonoids (apigenin, luteolin, kaempferol, quercetin, genistein, naringenin, phlorizin, and EGCG) on HCA profiles in roast beef patties cooked at 230°C for 20 min. Their findings show that most of these antioxidants significantly ($p < 0.05$) inhibited all HCAs investigated in the study (Harman, Norharman, 4, 8-MeIQx, MeIQx, DMIP, 1, 5, 6-TMIP, and PhIP). However, apigenin did not inhibit MeIQx formation, EGCG did not inhibit 1, 5, 6-TMIP formation,

and luteolin did not inhibit PhIP formation. Cheng et al. [11] showed that adding phenolic antioxidants such as quercetin, theaflavin 3, 3'-digallate, epicatechin gallate, rosmarinic acid, and naringenin to a chemical model system had no effect on PhIP, MeIQx, and 4, 8- DiMeIQx formation. However, when these antioxidants added to the surface of beef patties, the levels of PhIP, MeIQx, and 4, 8- DiMeIQx were significantly inhibited. Moreover, among these antioxidants, naringenin was the strongest inhibitor in both the model system and beef patties. Other research also found that adding phenolic compounds such as rutin and chlorogenic acid to roast beef patties significantly ($p < 0.05$) enhanced PhIP formation. One possible explanation for these differences may be that in model systems PhIP is produced using phenylalanine while MeIQx and 7, 8-diMeIQx model systems are produced using glycine [37]. Even though natural spices and flavonoid compounds did reduce PhIP formation, more research is needed to more thoroughly investigate these compounds and how they work.

Table 2. Effect of flavonoid compounds on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Flavonoid compounds (ppm)		PhIP ($\mu\text{g/ml}$) *	Inhibition (%)
Quercetin	Control	1.69 \pm 0.065	
	125	1.43 \pm 0.065	15
	625	1.43 \pm 0.065	15
	1250	1.19 \pm 0.065	30
Apigenin	Control	1.90 \pm 0.058	
	125	1.43 \pm 0.058	25
	625	1.60 \pm 0.058	16
	1250	1.62 \pm 0.058	15
Genistin	Control	1.79 \pm 0.091	
	125	1.17 \pm 0.091	35
	625	1.03 \pm 0.091	42
	1250	1.27 \pm 0.091	29
Phlorizin	Control	1.95 \pm 0.048	
	125	1.67 \pm 0.048	14
	625	1.70 \pm 0.048	13
	1250	1.60 \pm 0.048	18
Catechin	Control	2.23 \pm 0.067	
	125	1.74 \pm 0.067	22
	625	1.72 \pm 0.067	23
	1250	1.74 \pm 0.067	22

Significant differences were observed between control and treatments at $p < 0.05$.

*Means \pm standard error for each level of the treatment (n=3).

4. Conclusions

This study used chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour to inhibit the PhIP formation, which is classified by IARC as a potential carcinogen in protein-rich foods. The findings show that four out of five spices (black pepper oil, piperine, D-limonene, and capsaicin) did significantly ($p < 0.05$) reduce PhIP formation while P-cymene did not. All flavonoid compounds investigated in the study significantly ($p < 0.05$) reduced PhIP formation although genistin was

the strongest inhibitor while phlorizin had the least effect on PhIP generation. The effect of natural spices and flavonoid compounds on HCA formation in chemical model systems have not been studied extensively. Our results provide some insight into the effect of natural spices and flavonoid compounds on HCA formation, but we must still seek a better understanding of how natural spices and flavonoid compounds inhibit HCA formation, thus allowing us to minimize carcinogenic compounds in our daily foods.

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