

## Inhibitory Effect of Fruit Extracts on the Formation of Heterocyclic Amines

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Natural extracts have attracted considerable attention for development into effective inhibitors against the formation of genotoxic heterocyclic amines (HAs) in processed foods. In this study, four fruit extracts (apple, elderberry, grape seed, and pineapple) were evaluated for their effects on HA formation in fried beef patties. Apple and grape seed extracts were found to be the most effective in both the degree of inhibition in the formation of individual HAs (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and 2-amino-1-methyl-6-henylimidazo [4,5-*b*]pyridine (PhIP)) and in the reduction of total HA content (~70% relative to the control). Activity-guided analysis of apple extract using model systems (PhIP- and MeIQx-producing models) showed that the proanthocyanidins, phloridzin and chlorogenic acid were responsible for reducing the amount of HAs formed. Proanthocyanidins were identified as the dominant inhibitors because they were strongly active against HA formation in both the PhIP and MeIQx model systems. For phloridzin, the inhibitory effect was observed only on the formation of PhIP. In contrast, chlorogenic acid, although effective against the formation of MeIQx, significantly enhanced the formation of PhIP. This is the first report showing the inhibitory activities of apple phenolics on the formation of heterocyclic amines. The findings provide valuable information for the development of effective strategies to minimize HA content of cooked meats and to identify several new natural products that may have new applications in the food industry.

**KEYWORDS:** Genotoxic heterocyclic amines; fruit extracts; phloridzin, chlorogenic acid, polyphenolics, proanthocyanidins

### INTRODUCTION

A group of heterocyclic amines (HAs) are known to be formed during heating of meat and fish, with amino acids, sugars, and creatine as precursors (1–3). Although they are usually formed at very low concentrations (ppb), their presence suggests potential safety issues relative to their potent mutagenicity as shown in bacteria mutagenicity tests (4) and carcinogenicity in animal studies (5, 6). Moreover, epidemiological studies have indicated a positive correlation between consumption of well-cooked meats and development of certain cancers, especially those of the colon (7–9). Because these genotoxic substances are contained in primary foods and avoidance of their occurrence in most western diets is difficult, the public is therefore continuously exposed to HAs. Although HA-associated health risks also depend on many factors, including the bioavailability of HAs and genetic susceptibility, a long-term

strategy in the food industry is to seek ways to minimize or prevent their formation.

Time and temperature are among the most important parameters affecting HA formation. Prolonged heat treatment of muscle-based food at high temperature leads to higher HA content (10–12). It is thus advisable to avoid such cooking practice. The Maillard reaction has been known to contribute to the formation of HAs (3). Therefore, yields of HAs may be reduced by incorporating ingredients that can competitively react with their biochemical precursors (14, 15) or intermediates in the Maillard reaction such as pyridine, pyrazine, and reactive carbonyl species (3, 16). Because free radicals are considered to be involved in the formation of HAs (13), the addition of antioxidants has been one approach because these compounds may inhibit HA formation by scavenging free radicals (17, 18). A number of synthetic (18) and natural antioxidants (19) have been found to effectively reduce HA formation. However, several considerations arise regarding the value of using pure antioxidants in practical applications. First, they exhibit different effects on the formation of different HAs. For instance, ellagic acid produced an inhibitory effect on 2-amino-3,8-dimethylimi-

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dazo [4,5-*f*]quinoxaline (MeIQx) formation (19), but strongly enhanced the formation of 2-amino-1-methyl-6-henylimidazo [4,5-*b*]pyridine (PhIP). Additionally, there are concerns related to the cost, ease of accessibility, and adoption by the general public. These shortcomings may largely be overcome by the use of natural plant extracts that may contain a mixture of different types of phytochemicals. These phytochemicals may complement each other's activity to generate a more pronounced reduction in total HA content as compared to the use of a single compound. As examples, the inhibitory effects of spices, rosemary, thyme, sage, and garlic (20, 21), tea (22), tomato (23), and cherry tissue (24) on HA formation are recognized.

In recent years, fruit extracts have attracted increasing attention because they are rich in phytochemicals, and particularly in polyphenols, a class of compounds long associated with improvement in human health, nutrition, and with often strong antioxidant activity. Extracts of small berries, including grape, are among the most popular fruit extracts on the market. There has been strong scientific evidence supporting their activities in cardiovascular protection, antimicrobial infection, anti-inflammation, antiulceration, stress-reducing, antioxidation, and anticancer activity (25). Phenolic compounds, mainly stilbenes, anthocyanins, and proanthocyanidins, are believed to play important roles in providing the above health benefits (25). Apple extract has been shown to contain flavonoids, anthocyanins and proanthocyanidins, phenolic acids, lignans, and triterpenoids, which contribute to its antioxidant and anticancer properties (26–30). Pineapple fruit, popular in many world cuisines, has also been found to be a rich source of phytochemicals (31). In current study, extracts of apple, elderberry, grape seed, and pineapple were examined for their effects on HA formation in beef patties. Apart from identifying effective natural extract against HA formation, principal inhibitors in the apple extract were also identified by chromatographic and spectral methods. The findings suggest that apple proanthocyanidins are potent inhibitors of HA formation.

## MATERIALS AND METHODS

**Materials.** Amberlite XAD-16, catechin, chlorogenic acid, creatinine, epicatechin, Folin–Ciocalteu's phenol reagent (2N), gallic acid, glucose, phenylalanine, and phloridzin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Sephadex LH-20 was from GE Healthcare Biosciences AB (Sweden). Fresh ground beef was purchased from a local beef vendor (Hong Kong). Apple and grape seed extracts were obtained from BannerBio Nutraceuticals Inc. (Shenzhen, P.R. China). Elderberry extract was obtained from Naturex S. A. (Avignon, France). Pineapple extract was prepared in our laboratory. Procyanidin B1 and procyanidin B2 were purchased from Extrasynthese (Genay, France). HA standards, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), MeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo [4,5-*f*]quinoxaline (7,8-DiMeIQx), and PhIP were sourced from Toronto Research Chemicals (Toronto, Canada). Propyl-sulfonic acid (PRS) Bond-Elut cartridges (500 mg), C-18 cartridges (100 mg), Bond-Elut reservoir, and packing materials (diatomaceous earth) were from Varian Inc. (Harbor City, CA). All solvents used were of analytical grade and were obtained from BDH Laboratory Supplies (Poole, UK). The Reacti-Therm III heating module (Model 18840) and the screw cap Tuf-Bond Teflon fitted glass reaction vials (10 mL capacity) were purchased from Pierce Co. (Rockford, IL, USA).

**Preparation of Pineapple Extract.** Fresh pineapple was peeled, cut into small pieces, and blended using a Moulinex blender. From the resulting pineapple paste, 1 kg was extracted with 2 L 95% ethanol. The extraction process was repeated three times. Ethanol in the ethanol extract was then evaporated using a rotor evaporator under vacuum. The concentrated aqueous extract was then mixed thoroughly with 500 g

Amberlite XAD-16 that was previously washed with distilled water. This was allowed to stand under room conditions for 40 min before loading onto a glass column. Five bed volumes of Milli-Q water were used to wash away water-soluble components (primarily simple sugars) of pineapple juice. This was followed by elution with 5 bed volumes of 95% ethanol. The ethanol eluate was evaporated to dryness using a rotor evaporator. The powder obtained was stored in a desiccator.

**Total Phenolic Content of Fruit Extracts Determined by the Folin–Ciocalteu Assay.** Total phenolic content of the extracts was determined using the procedure proposed by Singleton and Rossi (32) with slight modifications. Briefly, fruit extracts were dissolved in DMSO and were diluted to appropriate concentrations using Milli-Q water. A 200  $\mu$ L portion of diluted sample was added into a cuvette. This was followed by the addition of 1 mL of FCR (1:10 diluted). After mixing, 0.8 mL of sodium carbonate solution (75 g/L) was added, and the reaction mixtures were allowed to incubate at ambient temperature for two hours before reading their absorbance at 765 nm. Quantitation was based on a standard curve obtained from a series of gallic acid solutions. Results were expressed as grams of gallic acid equivalent (GAE) per gram of fruit extract. Triplicate measurements were taken for each sample, and triplicate experiments were conducted.

**Evaluation of Effects of Fruit Extracts on HA Formation in Beef Patties.** An accurately weighed amount ( $30 \pm 0.2$  g) of ground beef was formed into a meat disk with the aid of a glass Petri dish ( $6.2 \times 1.2$  cm). Three samples were taken for each treatment in each experiment and triplicate analyses were performed. Briefly,  $30 \pm 0.2$  mg (0.1%) of each crude fruit extract was thoroughly mixed with the corresponding patties. The patties were then covered with Glad wrap and were allowed to incubate under ambient conditions for 60 min before frying. The patties were fried on a Teflon-coated pan at 210 °C for 6 min on each side (3 min  $\times$  2 for each side).

The three meat patties for each treatment were homogenized for 2 min in 150 mL of 1 M NaOH to a dense paste. An amount equivalent to one-sixth of the total weight of the paste was weighed into a beaker. Next, 25 mL of 1 M NaOH was added to the paste, and the resultant suspension was sonicated for twenty minutes. Extraction recoveries were determined by spiking selected samples with 50  $\mu$ L of standard HA solution of known concentrations. The suspension was then thoroughly mixed with diatomaceous earth and then packed into empty reservoirs fitted with a bottom frit (20  $\mu$ m). Because of the large sample size, three reservoirs were required for complete packing of each mixture. All together, 300 mL of dichloromethane containing 5% toluene was used for each sample (100 mL for each packed reservoir). Eluate from the three reservoirs was combined before eluting through a conditioned PRS cartridge. The PRS cartridges were then dried under maximum vacuum for 5 min and were sequentially washed with 6 mL of 0.1N HCl, 15 mL of 40% methanol in 0.1N HCl, and 2 mL of water. HAs retained were then eluted into Bond-Elut C-18 cartridges with 20 mL of ammonium acetate solution (0.5 M, pH 8). Prior to use, the PRS and C-18 cartridges were conditioned according to a method suggested by Gross and Gruter (33). The C-18 cartridges were washed with 2 mL of water and were dried under positive pressure. Final elution was carried out with 1.2 mL of MeOH-NH<sub>4</sub>OH (9:1, v/v) into microvials. The eluate was dried under nitrogen gas, and the residue was dissolved in 100  $\mu$ L of methanol for HPLC analysis.

**Evaluation of Effects of Apple-Extract Fractions on HA Formation in Model Systems.** A PhIP-producing model and a MeIQx-producing model were used to compare the effects of different fractions from apple extract on HA formation. The PhIP-producing system contained 0.4 mmol of phenylalanine, 0.4 mmol of creatinine and 0.2 mmol of glucose, as reported previously (19, 34). Each fraction ( $30 \pm 0.2$  mg powders) was added directly into the reaction vials. The reaction medium was 3 mL of diethylene glycol containing 14% water. For the MeIQx-producing model, 0.4 mmol of glycine was used in place of phenylalanine, and all other reactants were the same. The vials were sealed with screw caps fitted with Tuf-Bond Teflon. Three milliliters of silicone oil were added to each cavity of the heating block. The temperature meter of the heating block was set to 128 °C and was preheated for 2 h prior to inserting the vials into the cavities. Total heating time was 2 h, with the temperature checked every 20 min. The temperature fluctuation was within 2 °C. The vials were immediately

**Table 1.** Total Phenolic Content of Fruit Extracts Determined by Folin-Ciocalteu Assay and Effect of These Extracts on the Formation of HAs in Beef Patties Fried at 210 °C for 6 min on Each Side<sup>a</sup>

treatment	total phenolic content (g of gallic acid per g of extract)	heterocyclic amines (ng/g beef patties) and inhibition (%)			
		PhIP	4,8-DiMeIQx	MeIQx	Total (HAs)
control		10.10 ± 0.85 a	0.95 ± 0.11 a	2.96 ± 0.49 a	14.01
grape seed	0.88 ± 0.04	2.80 ± 0.40 b (72)	0.32 ± 0.07 b (66)	1.00 ± 0.41 b (67)	4.13 (71)
apple	0.82 ± 0.03	3.14 ± 0.16 b (69)	0.34 ± 0.07 b (63)	1.24 ± 0.38 b (59)	4.72 (66)
elderberry	0.24 ± 0.01	5.54 ± 0.49 b (45)	0.76 ± 0.13 a (19)	2.80 ± 0.76 a (6)	9.10 (35)
pineapple	0.10 ± 0.00	8.85 ± 0.11a (13)	0.77 ± 0.08 a (18)	2.23 ± 0.96 a (27)	11.86 (15)

<sup>a</sup> Mean ± standard deviations; *N* = 3 for all treatments.

cooled in an ice-water mixture at the 2 h time point. The content of each vial was mixed with 57 mL of 2 M NaOH. A 5 mL diluted aliquot was transferred into a 100-mL beaker. The aliquots were mixed thoroughly with diatomaceous earth, and subsequent solid phase cleaning steps were similar to those applied to beef patty samples except that only 48 mL of dichloromethane containing 5% toluene was used for extracting HAs from diatomaceous earth.

**HPLC Analysis of HAs.** HPLC analysis was performed using a Shimadzu HPLC system with a LC-20AT separation module, a SIL-20A autosampler, a DGU-20A<sub>3</sub> degasser, and a SPD-M20A photodiode array detector. Separation of HAs was carried out on an YMC-Pack Pro C-18 column (5 μm, 150 × 4.6 mm i.d., Waters Corporation, Milford, MA, USA). The elution program was adopted from Shin and coworkers (14) with slight modifications. The mobile phase was a water (0.01 M triethylamine phosphate, pH 3.2)/acetonitrile gradient with a flow rate of 1 mL/min. The initial ratio of water (triethylamine phosphate, 0.01 M, pH 3.2)/acetonitrile was 95:5, which was changed to 83:17 during the first 10 min. The acetonitrile concentration continued to linearly increase up to 75:25 for the next 10 min, and then the concentration was linearly increased to 55:45 during the following 10 min. Finally, the acetonitrile concentration was increased to 20:80 in 5 min. The total running time was 35 min, and the postrunning time was 15 min for equilibration of the column. Target HAs were monitored at wavelengths of 265 and 312 nm, respectively. Before commencing an analytical run, the column was conditioned with the initial mobile phase composition for 30 min, and the LC-PDA system was tested for system stability using blank and standard HA solutions. Peak identification was accomplished by comparing the retention times and UV spectral characteristics of the HPLC peaks with those obtained from standard solutions of HA mixture analyzed under the same conditions. Quantitative determination was performed using an external calibration curve. Correlation coefficients (*r*<sup>2</sup>) for HA standard curves were 0.9996 for PhIP, 0.9968 for MeIQx, and 0.9999 for 4,8-DiMeIQx. Triplicate analyses were performed for each treatment.

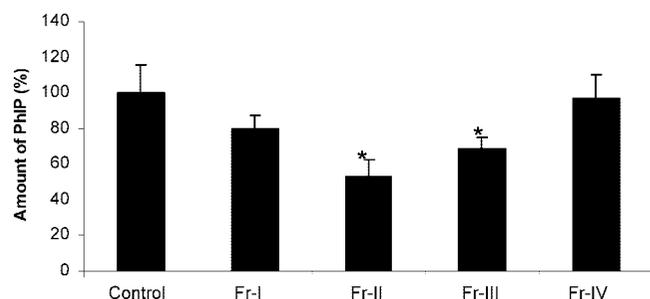
**Fractionation of Apple Extract and Identification of Inhibitors of HA Formation in Apple.** Crude apple extract (22 g) was dissolved in Milli-Q water and was loaded onto an Amberlite XAD-16 column (40 × 4 cm). Elution was performed using ethanol-water containing 0, 10, 30, 50, 70, and 95% ethanol, respectively, each with 500 mL, which was collected in two 250 mL fractions. The profile of each fraction was checked using HPLC, and similar fractions were combined. This resulted in several pooled fractions that were then dried using a rotor evaporator under reduced pressure. In total, four pooled fractions Fr-I-IV were collected for examining the effect on HA formation. Fractions Fr-II and Fr-III, which showed effective inhibition of PhIP formation, were further analyzed. Fr-III was found to be a single substance and was directly subjected to LC-MS analysis. Because Fr-II contained multiple components, Sephadex gel column chromatography was applied for further separation. Briefly, 2 g of Fr-II extract was dissolved in 70% methanol and was loaded onto a conditioned Sephadex LH-20 column (40 × 4 cm). The mobile phase was 70% methanol/30% water, and the eluate was collected at 10 min intervals with a fraction collector. Each fraction had a volume of about 15 mL. This chromatographic process led to three subfractions (Sub-fr1-3). Sub-fr2 was found to contain a dominant component of 98% purity using HPLC analysis. Sub-fr2, together with sub-fr3, a multicomponent fraction, were subjected to LC-MS for structural identification.

Negative and positive electrospray ionization (ESI)-mass spectra were measured using an Agilent LC-MSD system (Agilent Technologies, Germany) equipped with an electrospray source, Bruker Daltonics 4.0, data analysis version 4.0 software, and a Hewlett-Packard 1100 HPLC system with an autosampler, a quaternary pump system, a photodiode array detector, and a HP Chemstation data system. A prepacked 150 × 3.2 mm prodigy ODS3 column (5 μm particle size, Phenomenex, Torrance, CA) was selected for LC-MS analysis. The mobile phase was a linear gradient of water with 0.2% formic acid (A)/acetonitrile (B): 0 min, 94% A, 6% B; 4 min, 94% A, 6% B; 20 min, 84% A, 16% B; 30 min, 70% A, 30% B; and 40 min, 30% A, 70% B. Postrunning time was 10 min. The ESI-MS was operated under positive or negative ion mode with an optimized collision energy level of 20%. The mass scan range was from *m/z* 100 to 1600. ESI was conducted using a needle voltage of 3.5 kV. High-purity nitrogen (99.999%) was used as dry gas at a flow rate of 12 L/min, with a capillary temperature at 325 °C. Helium (60 psi) was used for nebulization. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity.

**Statistical Analysis.** Statistical analyses were performed using the SPSS statistical package (SPSS, Inc., Chicago, IL). Paired samples *t*-test was applied to determine whether a particular treatment of the sample would result in significantly different content of HAs as compared with the control. Treatment differences with *P* < 0.05 were preset to indicate whether means were significantly different.

## RESULTS AND DISCUSSION

**Effect of Natural Extracts on Formation of HAs in Fried Beef Patties.** Natural extracts usually contain a mixture of various types of phytochemicals, which likely exhibit different mechanisms of interaction with regard to the formation of different HAs. Greater reductions in total HA contents of the final food products could arise from the use of these extracts rather than the use of a single pure phytochemical. In this study, four fruit extracts were evaluated for their effect on the formation of HAs in beef patties fried at 210 °C for 6 min on each side. HPLC analysis was carried out only for the amounts of polar HAs formed relative to the control because these HAs account for most of the HA-associated mutagenic/carcinogenic activity in foods (35). Three polar HAs, including MeIQx, 4,8-DiMeIQx, and PhIP were identified with corresponding yields of 2.96 ± 0.49, 0.95 ± 0.11, and 10.10 ± 0.85 ng/g, respectively. Average recovery using solid phase extraction was 65% for MeIQx, 47% for 4,8-DiMeIQx, and 64% for PhIP. Among all of the HAs, PhIP formed in the highest quantity, and this is in agreement with previous reports (10, 36, 37). As shown in Table 1, except for pineapple extract, the grape seed, apple, and elderberry extracts at 0.1% were capable of significantly (*P* < 0.05) reducing the level of PhIP formed by 72%, 69%, and 45%, respectively. However, for the levels of MeIQx and 4,8-DiMeIQx, only grape seed and apple extracts resulted in significant reductions (*P* < 0.05). At 0.1%, grape seed and apple extracts reduced the formation of MeIQx by 67% and 59%, respectively, and that of 4,8-DiMeIQx by 67% and 62%,

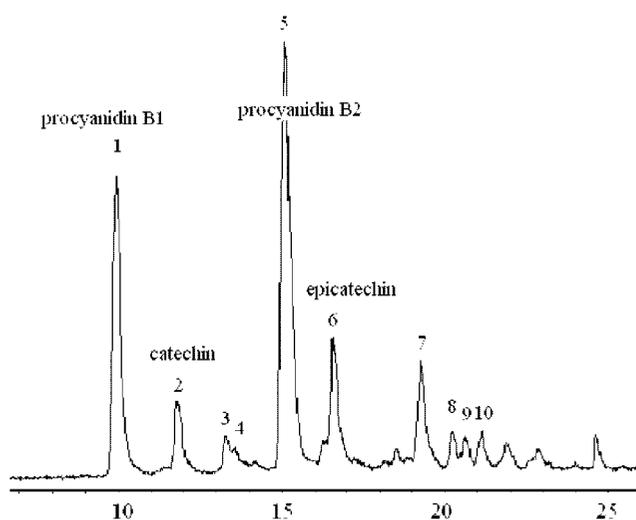


**Figure 1.** Relative percentages of the amounts of PhIP formed in a chemical model system containing creatinine, glucose, and phenylalanine with each fractions of apple extract. All treatments were run in triplicate. Bars with an asterisk indicate a significant difference from the control at  $P < 0.05$ .

respectively. Thus, both grape seed and apple extracts exhibited comparable inhibitory effects with respect to each of the three polar HAs identified. The degree of reduction in the total HA content was another criterion used for selection of the most effective extract for further investigation. On the basis of such criteria, grape seed and apple extracts were again found to be the most effective against HA formation. Our results for grape seed are also in agreement with others reporting that grape seed extract significantly reduced the formation of polar and nonpolar HAs (38).

As in previous studies (38, 39) researchers attributed the inhibitory effects of several natural extracts on HA formation to their phenolic components; in the current study, we also quantified the total phenolic contents (Table 1) of the four extracts to examine whether this parameter correlated with the potency of inhibition in HA formation. A good positive correlation was observed providing supportive evidence that the inhibitory activity of these extracts is at least partially due to their phenolic contents. However, whether this is via the scavenging of free radicals, reactive carbonyl species, or other degradation products from the precursors (3, 13, 40, 41) requires further mechanistic studies. Prior to such mechanistic investigation, dominant inhibitors in the extract of interest would need to be identified. Because there has been previous report on the inhibitory activity of grape seed extract in HA formation, only apple extract was chosen for further investigation.

**Identification of Dominant Inhibitors of HA Formation from Apple Extracts.** Two models, a PhIP-producing and a MeIQx-producing model were used to monitor the inhibitory activities of fractions from apple extract on HA formation. The yield of PhIP was  $40.0 \pm 8.67$  nmol/mmol creatinine, and that of MeIQx was  $21.6 \pm 2.70$  nmol/mmol creatinine, in these two model systems. Amberlite XAD-16 treatment of crude apple extract gave rise to four major fractions (Fr-I–IV). Because of a limited amount of one of the fractions, identification of the most active fraction for inhibition of HA formation was only performed using a PhIP-producing model because PhIP is the dominant HA formed in meat products. As shown in Figure 1, only Fr-II and Fr-III were effective against the formation of PhIP. Fr-III was identified as phloridzin by interpretation of LC-MS data and by comparing its retention time and UV-absorption spectrum with those acquired from standard phloridzin. In positive ESI-MS, a molecular ion at  $m/z$  459.4  $[M + Na]^+$  and the protonated aglycone ion  $[M + H]^+$  at  $m/z$  275.5 were clearly observed. In the HPLC chromatograms, Fr-III showed an identical retention time and UV absorption as phloridzin. Fr-II was further separated by a Sephadex LH-20 column into three subfractions, which were then compared for their effects on the formation of PhIP in the model system (data not shown). Sub-



**Figure 2.** Total ion chromatogram of sub-fr3 of apple extract under negative ESI-MS mode.

fr3 exerted a strong inhibitory effect on the formation of PhIP. In contrast, sub-fr2 significantly ( $P < 0.05$ ) enhanced PhIP formation. Sub-fr2 was found to contain a single component, which was also one of the major constituents in the apple extract we selected for examination. In positive ESI-MS, sub-fr2 showed significant molecular ion peaks at  $m/z$  355.3  $[M + 1]^+$  and 377.2  $[M + Na]^+$ ; whereas in negative ESI-MS it showed a molecular ion peak at 353.1  $[M - 1]^-$ , which is assignable to the structure of chlorogenic acid. The identity of sub-fr2 was further confirmed by comparison of LC retention time and the UV-spectrum with that of authentic chlorogenic acid. Attempts to separate the compounds in sub-fr3 were unsuccessful. LC-UV and LC-MS analyses were performed to characterize the phytochemical composition of sub-fr3 from Fr-II. Recording UV-vis absorption spectrum of the chromatographic peaks allowed their assignments to a certain class of polyphenols: proanthocyanidins, because all of the major peaks had nearly identical UV-absorption spectrum with maximum UV absorption at around 278 nm, similar to that of catechin. In the total ion chromatogram of negative ESI-MS (Figure 2), over 10 peaks were observed, with molecular ion peaks at 577.6  $[M - 1]^-$  (dimer), 289.5  $[M - 1]^-$  (monomer), 865.8  $[M - 1]^-$  (trimer), 865.7  $[M - 1]^-$ , 577.3  $[M - 1]^-$ , 289.4  $[M - 1]^-$ , 865.8  $[M - 1]^-$ , 577.5  $[M - 1]^-$ , 1153.8  $[M - 1]^-$  (tetramer), and 865.7  $[M - 1]^-$  for peaks 1–10, respectively. These peaks were assignable to proanthocyanidin monomers, dimers, trimers and tetramers. Four standard compounds, catechin, epicatechin, and procyanidins B1 and B2 were matched against the proanthocyanidins in this subfraction using HPLC. Peaks 1, 2, 5, and 6 were thus elucidated as procyanidin B1, catechin, procyanidin B2, and epicatechin, respectively (structures are shown in Figure 3).

Considering a previous study (19), which found that for some phenolics completely different effects (enhancing versus inhibitory) could be observed on the formation of different types of HAs in model systems, chlorogenic acid, phloridzin, and sub-fr3 of Fr-II were further examined for their effects on the formation of MeIQx in model systems. Figure 5 presents the relative activities of these apple components with regard to the formation of PhIP and MeIQx, respectively. As mentioned earlier, in the PhIP-producing model, sub-fr3 of Fr-II caused the greatest reduction ( $48.6 \pm 5.8\%$ ) in the level of PhIP formed relative to the control. Phloridzin also significantly ( $P < 0.05$ ) inhibited the formation of PhIP. Chlorogenic acid was proven

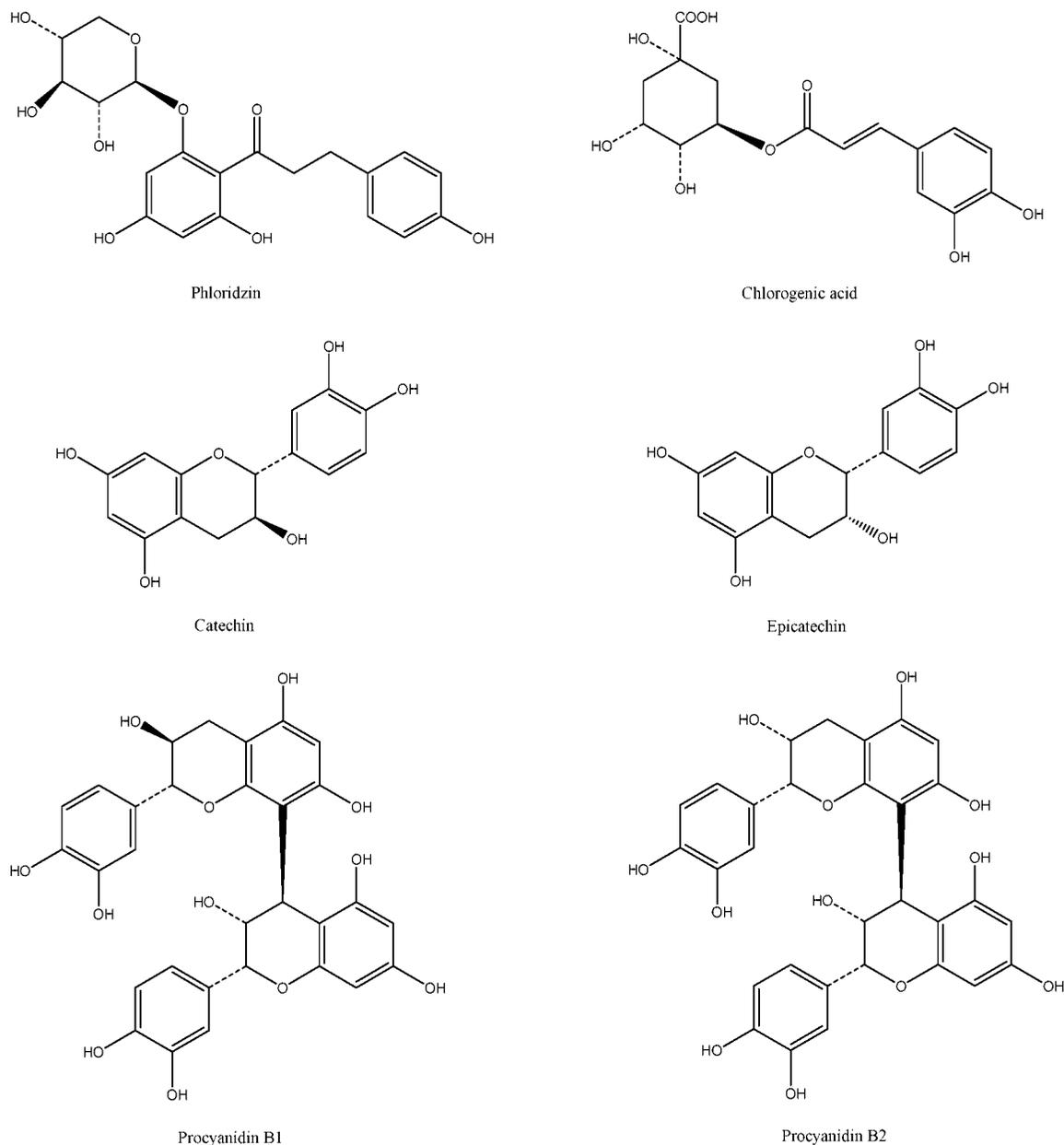


Figure 3. Structures of phenolics identified in apple extract.

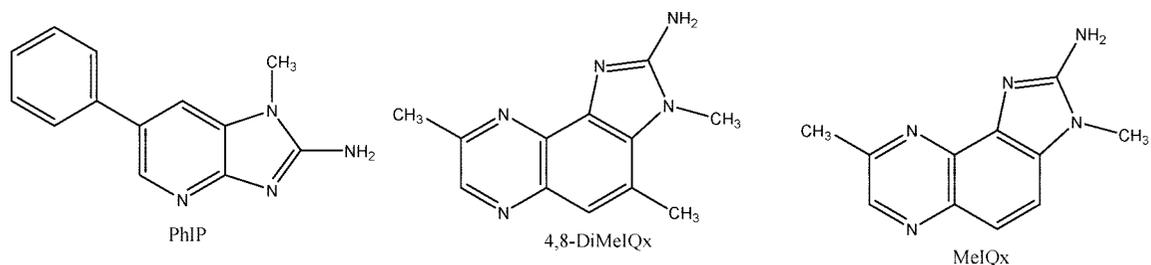
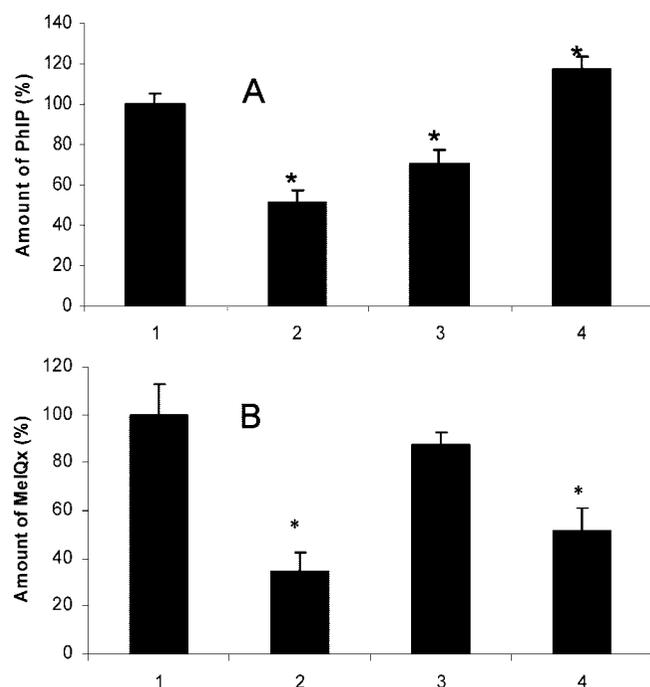


Figure 4. Structures of three HAs identified in fried beef patties.

to enhance PhIP formation. For the MeIQx model test, a different phenomenon was observed. Sub-fr3 showed significant inhibitory activity on MeIQx formation. Phloridzin did not significantly affect the level of MeIQx formed, whereas chlorogenic acid showed potent inhibitory activity as comparable to that of sub-fr3. Previous studies proposed that PhIP (42, 43) and MeIQx (13, 40) predominantly resulted from different reaction pathways. It is thus rational to expect that phloridzin and chlorogenic acid may complement each other's activity when applied together to systems capable of

producing different types of HAs. On the other hand, the above studies led us to the postulation that sub-fr3 (proanthocyanidin-rich) from Fr-II of the apple extract is the key inhibitor of HA formation. This fraction was effective in reducing the formation of both PhIP and MeIQx in model systems. The underlying mechanism(s) whereby certain proanthocyanidins could interrupt the formation of both PhIP and MeIQx, whereas phloridzin and chlorogenic acid only interrupt the formation of one of them, warrants further investigation.



**Figure 5.** Relative percentages of the amounts of PhIP (A) and MeIQx (B) formed in the chemical model system containing creatinine, glucose, and phenylalanine (A) or glycine (B) with each apple-extract components. (1) control; (2) sub-fr3; (3) phloridzin; (4) chlorogenic acid. All treatments were run in triplicate. Bars with an asterisk indicate significant difference from the control at  $P < 0.05$ .

There have been previous studies demonstrating the inhibitory activity of catechins from green and black teas on the formation of PhIP and MeIQx (19, 22). However, such findings have limited values in practical applications because tea extracts are rarely used in preparation or cooking of meats. This present study is the first to report the potency of apple proanthocyanidins in suppressing the formation of HAs. Thus, the incubation of meats with apple extract before cooking could potentially reduce HA load of the final products.

In conclusion, incubation of beef with selected natural extracts (0.1% by weight) before frying can reduce the formation of HAs. Grape seed and apple extracts strongly inhibited the formation of polar HAs in fried beef. Activity-guided analysis of apple extracts showed that the proanthocyanidins, phloridzin and chlorogenic acid play central roles in interfering with the reaction network involved in the formation of these HAs. Among these bioactive natural compounds, only the proanthocyanidins were able to inhibit the formation of both PhIP and MeIQx in the corresponding model systems. The identification of dominant inhibitors would facilitate selective concentration in favor of these phytochemicals during preparation of natural extracts intended for use in meat preparation and cooking. The use of these culinary natural extracts could significantly reduce dietary exposure to genotoxic heterocyclic amines.

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