Investigating the Synergetic Effect of Various Natural Antioxidants to Inhibit 2-amino-1-methyl-6-phenylimidazo [4,5-b] Pyridine (PhIP) Formation in Model Systems

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Abstract Natural antioxidants have many biological functions and serve as antioxidant and anti-inflammatory agents. Although the antioxidant effects of many spices and flavonoid compounds on 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) formation have been evaluated, research related to the synergistic antioxidant effect of various spices and flavonoids on PhIP formation is not well studied. In addition, at least some research shows a combination of compounds inhibits HCAs more strongly than a single antioxidant. Therefore, in this study, binary combinations of two antioxidant spices like piperine and capsaicin and two flavonoid compounds like genistin and catechin were investigated using a chemical model system that contained glucose, creatinine, and phenylalanine in 90:10 diethylene glycol/water (v/v) and heat-treated at 180°C for 1 hour to test the formation of PhIP. The PhIP contents were assessed using high-performance liquid chromatography (HPLC). All ratios of mixed spices and corresponding flavonoid compounds were as follows: 1:0.25, 1:0.5, and 1:1. The synergistic effect was assessed by identifying the reduction percentage of PhIP formation. All investigated combinations significantly (p< 0.05) reduced PhIP formation. The combination of piperine and genistin had the highest synergistic effect for all combinations. While the combination of catechin and capsaicin had the lowest synergistic effect. Knowing the antioxidants with the best synergistic effects could be useful in developing dietary antioxidants, leading to lower HCA formation.

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

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1. Introduction

Several studies have defined heterocyclic amines (HCAs) as mutagenic and carcinogenic compounds produced when high protein foods are cooked at high temperatures [1,2]. Based on Salmonella/mutagenicity test, more than 25 HCAs have been identified in cooked foods [3,4]. The International Agency for Research on Cancer (IARC) lists some of these as probably human carcinogens and some as possible human carcinogens [5]. One of the most abundant HCAs formed in cooked meat and fish during normal cooking is PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) [6]. Animal studies have found that food rich in PhIP increases the risk of prostate, breast, intestine, and liver cancers. It is also linked with an increase in cancer risk in humans [7]. Many strategies have been identified for reducing the potential health risk of HCAs in our foods, including reducing cooking times and temperatures and food marinades. Adding antioxidants is another effective treatment to limit HCA formation because of antioxidants have free radical scavenging properties [8].

In recent years, natural products that promote health and fitness have received much attention especially because of their antioxidant proprieties [9]. Antioxidant spices are widely used because they enhance flavor, color, and aroma in our daily foods. Spices do have, moreover, beneficial health effects [10]. Black and red pepper spices protect against liver and kidney diseases [11]. Free radicals normally produced in our bodies as a result of oxidation have a correlation with chronic diseases such as cancer, cardiovascular disease, diabetes, pulmonary and neurological diseases [12,13]. Black and red pepper spices, however, have free radical scavenging abilities and immunomodulatory properties which lead to protect the body from infectious diseases [14].
Flavonoids are a group of phenolic compounds widely found in nature with many biological functions; they are antioxidants, anti-inflammatory agents, and antimicrobial agents [15,16]. They are now receiving much attention from consumers and in food industries because they can protect against oxidative stress [17]. Various dietary flavonoid compounds such as apigenin, epigallocatechin gallate, genistein, kaempferol, luteolin, phlorizin, and quercetin have been investigated for their ability to inhibit HCA formation, especially PhIP. Other studies have revealed that these compounds significantly reduce HCA formation [18].

The synergistic effect among antioxidants has become increasingly important because of consumer interest in preserving nutritional value and positive effects on health [19]. Synergistic effects occur when two compounds interact, producing more inhibitory effect than the individual compounds [17]. Synergistic antioxidant effects have been reported in previous studies [20,21,22]. For instance, Hajimehdipoor, Shahrestani, and Shekarchi [23] investigated the synergistic antioxidant effects between phenolic compounds such as caffeic acid, gallic acid, and chlorogenic acid, and flavonoids such as rutin and quercetin. The results showed that caffeic acid and gallic acid had the most potent synergistic effect among all combinations of the compounds. The synergistic antioxidant effect was also found in the presence of iron [24], quercetin 3-β-glucoside [25], and some herbs [26]. However, interaction among antioxidants in addition to other additives can also lead to antagonistic effect [27]. Becker, Ntouma, and Skibsted [21] evaluated α-T, astaxanthin, quercetin, and rutin for their synergistic antioxidant effects. The results showed the factors that affect synergism and antagonism of antioxidants: solubility, polarity, and the hydrophilic nature of the antioxidants. Despite research into the individual effects of several spices such as piperine and capsaicin and natural flavonoids such as quercetin, apigenin, genistin, phlorizin, and catechin on PhIP formation [18,28,29], no study has investigated the synergistic effect among these antioxidants. Moreover, a combination of antioxidants would perhaps help reduce HCAs in our daily foods and positively affect health. Therefore, the aim of this study was to uncover the synergistic effects of two spices, piperine and capsaicin, when they interacted with two natural flavonoids, genistin and catechin, in binary combinations in a chemical model system.

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). Antioxidants flavonoids (genistin and catechin) and spices (piperine and capsaicin) standards were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). D-glucose (99.5%), L-phenylalanine (98%), creatinine, diethylene glycol, and trimethylamine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Solvents and chemicals such as acetic acid, acetonitrile (high-performance liquid chromatography [HPLC] grade), methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was processed by a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc, Dubuque, IA, USA). 0.2 µm syringe filters were provided by Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Preparation of Model Systems

The effects of antioxidant flavonoids and spices on PhIP formation were evaluated using a model system with slight modifications [30]. 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. 10 mg of flavonoids and spices (genistin, catechin, piperine, and capsaicin) were added to the model systems. Samples without flavonoids and spices were used as control. All ratios of mixed spice compounds as they corresponded to flavonoids were as follows: 1:0.25, 1:0.5, and 1:1. The reaction substances were added to a 1 ml reaction vial, which were then inserted into brass vessels with 2 screw caps on the top and bottom and 4 holes (1 cm x 1 cm) on the body. The brass vessels were then inserted into a heating block (HP 5890; Agilent Technologies, Inc., Santa Clara, CA, USA), and heated at 180°C for 1 hour and then immediately cooled by placing the reaction vials on ice for 5 min. All model system samples were syringe filtered and diluted 1:10 with methanol before HPLC analysis.

2.3. Analysis of PhIP

HPLC separation was carried out on an HP 1050 series HPLC (Agilent Technologies) coupled with an HP 1050 series diode array UV-visible detector and an HP 1046 fluorescence detector. Separation of PhIP in the model system samples was performed using reversed-phase chromatography using a TSKgel ODS-80TM (4.6 mm x 25 cm x 5µm) column and a TSK guardgel ODS-80TM (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan). The injection volume for each sample and the mobile phase rate were 20 µL and 1 ml/min, respectively. The mobile phase was composed of acetonitrile (solvent B) and 0.01 M trimethylamine (pH was adjusted to 3.6 with acetic acid) (solvent C). The mobile phase gradients were used as described previously [31] with minor modifications. The initial ratio of a linear HPLC gradient started with 95% C and 5% B and then decreased to 75% C and 25% B over 30 minutes. After 35 min, the initial ratio of 95% C and 5% B was maintained for 4 min to equilibrate of the column before the next injection. For PhIP detecting, fluorescence detector was setting at emission/ excitation wavelengths of 437 nm and 229 nm.

2.4. Quantification and Statistical Analysis

A standard method was performed to quantify PhIP in the model system. 1 mg of PhIP dissolved in 4 ml of methanol and gradually diluted to 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.007813 ppm (Appendix A). A standard curve was processed after determining
3. Results and Discussion

Antioxidant spices and flavonoids interact in several different ways to inhibit HCA formation. Combinations of these antioxidants can produce higher reductions of HCAs in our daily foods than single antioxidants [30]. In this study, a binary combination of antioxidant spices (piperine and capsaicin) and the flavonoid compounds (catechin and genistein) were evaluated for their ability to reduce PhIP formation in a chemical model system heated at 180ºC for 1 hour. HPLC analysis was performed to determine how much PhIP was generated in the model system. The combined ratio of antioxidant spices and flavonoids were as follows: 1:0.25, 1:0.5, and 1:1. As expected, all combinations of antioxidants spieces and flavonoids evaluated in this study significantly (p < 0.05) reduced PhIP formation, indicating their various antioxidant properties. The results indicated that the combined effect of piperine and catechin (with piperine at higher levels than catechin) was the strongest, ranging from 26% to 41% reduction compared to control group (see Table 1). The synergistic effect increased as the concentrations of the combined compounds increased, indicating that piperine and catechin combined might be the most potent inhibitor of PhIP formation. The combination with higher levels of catechin and lower levels of piperine showed a lower synergistic effect with reduction ranging from 17% to 22% (see Table 1).

Table 1. Combined effect of piperine and catechin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180ºC for 1 hour

| Piperine + Catechin | PhIP (µg/ml) * | Inhibition (%) |
|---------------------|----------------|----------------|
| Control             | 2.03 ± 0.091   |                |
| 1:0.25              | 1.51 ± 0.091   | 26             |
| 1:0.5               | 1.49 ± 0.091   | 27             |
| 1:1                 | 1.20 ± 0.091   | 41             |

| Catechin + Piperine |
|---------------------|
| Control             | 2.00 ± 0.061 |
| 1:0.25              | 1.67 ± 0.061 | 17 |
| 1:0.5               | 1.58 ± 0.061 | 21 |
| 1:1                 | 1.56 ± 0.061 | 22 |

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

Table 2 shows piperine and genistein (with piperine at higher levels than genistein) was the strongest. In contrast, combining higher levels of genistein and lower levels of piperine showed less effect. In fact, the effect of the synergistic interactions decreased as the concentrations of the combined compounds increased, although these results do show that piperine, when combined with other compounds, exhibits a high percentage of inhibition (see Table 2). Our results agree with previous results that demonstrated piperine, when combined with spices like curcumin (a bioactive compound of turmeric), a potent synergistic effect occurs. This is probably because piperine is one of the most active antioxidants reported to inhibit P-glycoprotein. Which is a protein responsible for transporting substances outside the cell membrane. P-glycoprotein is present in brain circulation, which might change the beneficial effects of other antioxidants such as like curcumin. Thus, piperine has a potent synergistic antioxidant effect through inhibiting the P-glycoprotein molecule. Piperine can also help other compounds by increasing their absorption [32]. Nimkar and Smith [33] investigated the antioxidant interactions between black pepper and other spices such as rosemary, cinnamon, oregano, turmeric, thyme, and ginger on the inhibition of PhIP formation in beef patties. Significant synergistic effects against PhIP formation were observed, with the highest synergistic effect between black pepper and turmeric (94.7% inhibition).

Table 2. Combined effect of piperine and genistein on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180ºC for 1 hour

| Piperine + Genistein | PhIP (µg/ml) * | Inhibition (%) |
|----------------------|----------------|----------------|
| Control              | 2.64 ± 0.065   |                |
| 1:0.25               | 2.08 ± 0.065   | 21             |
| 1:0.5                | 2.27 ± 0.065   | 14             |
| 1:1                  | 2.27 ± 0.065   | 14             |

| Genistein + Piperine |
|----------------------|
| Control              | 1.97 ± 0.044 |
| 1:0.25               | 1.61 ± 0.044 | 18 |
| 1:0.5                | 1.75 ± 0.044 | 11 |
| 1:1                  | 1.67 ± 0.044 | 15 |

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

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

Table 3 shows the combined effects of capsaicin and catechin (with capsaicin at higher levels than catechin). The synergistic effect was equal among all levels of the combination. Combinations of higher levels of catechin and lower levels of capsaicin, however, exhibited less reduction against PhIP formation.

Synergistic effect of four antioxidants, including capsaicin, vitamin E, quercetin, and ascorbic acid have also been evaluated. The results have shown that capsaicin has a potent synergist effect [34]. On the other hand, a combination of different types of antioxidants can lead to antagonist effects, where individual effects overpower the combined effects [35]. For instance, ternary combinations of rutin, caffeic acid, rosmarinic acid; chlorogenic acid, caffeic acid, rosmarinic acid; rutin, rosmarinic acid, gallic acid; and rutin, chlorogenic acid, caffeic acid showed significant antagonist effects, reducing their ability to inhibit HCA formation by approximately 16 to 22% [23]. Zeng, Li, He, Qin, and Chen [36] investigated the synergistic or antagonist effect of phenolic compounds such as rutin and protocatechuic acid on HCA...
profiles in roast beef patties. Their findings indicated that combinations of these compounds had significant ($p < 0.05$) synergistic effects against harman and norharman-type HCAs, but significant ($p < 0.05$) antagonistic effects were observed for DMIP and 4, 8-DiMeIQx-type HCAs.

**Table 3. Combined effect of capsaicin and catechin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180ºC for 1 hour**

| Capsaicin + Catechin | PhIP (µg/ml) * | Inhibition (%) |
|----------------------|----------------|----------------|
| Control              | 2.09 ± 0.053   |                |
| 1:0.25               | 1.69 ± 0.053   | 19             |
| 1:0.5                | 1.69 ± 0.053   | 19             |
| 1:1                  | 1.69 ± 0.053   | 19             |

| Catechin + Capsaicin | PhIP (µg/ml) * | Inhibition (%) |
|----------------------|----------------|----------------|
| Control              | 2.55 ± 0.051   |                |
| 1:0.25               | 2.20 ± 0.051   | 14             |
| 1:0.5                | 2.21 ± 0.051   | 13             |
| 1:1                  | 2.18 ± 0.051   | 15             |

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

$^{*}$Means ± standard error for each level of the treatment ($n=3$).

Finally, **Table 4** shows the combined effect of capsaicin and genistin (with capsaicin at higher levels than genistein) was the strongest. In contrast, combinations with genistein at higher levels and capsaicin at lower levels showed reduced PhIP formation. This result suggests that lower concentrations of capsaicin and genistein combined reduced PhIP formation more than higher concentrations (see **Table 4**).

**Table 4. Combined effect of capsaicin and genistin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180ºC for 1 hour**

| Capsaicin + Genistein | PhIP (µg/ml) * | Inhibition (%) |
|-----------------------|----------------|----------------|
| Control               | 2.07 ± 0.052   |                |
| 1:0.25                | 1.66 ± 0.052   | 20             |
| 1:0.5                 | 1.71 ± 0.052   | 17             |
| 1:1                   | 1.72 ± 0.052   | 17             |

| Genistein + Capsaicin | PhIP (µg/ml) * | Inhibition (%) |
|-----------------------|----------------|----------------|
| Control               | 2.07 ± 0.042   |                |
| 1:0.25                | 1.71 ± 0.042   | 17             |
| 1:0.5                 | 1.77 ± 0.042   | 15             |
| 1:1                   | 1.79 ± 0.042   | 15             |

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

$^{*}$Means ± standard error for each level of the treatment ($n=3$).

### 4. Conclusion

All combinations of antioxidant spices (piperine and capsaicin) and flavonoids (genistein and catechin) had significant synergistic effects, with combinations of piperine and genistein having the highest synergistic effect. Combinations of capsaicin and catechin had the least synergistic effect. The results of this study show all tested antioxidants have a synergistic effect in reducing PhIP formation in a chemical model system, but future studies could determine how applying antioxidant spices and flavonoid compounds to beef patties would be helpful to promote human consumption of meat products with fewer HCAs.

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