Cytotoxic effects of peanut phenolic compounds possessing histone deacetylase inhibitory activity on human colon cancer cell lines

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Abstract: Phenolic compounds present in our diet play an important role in colon cancer chemoprevention. Previous results demonstrated that peanut testa extract inhibited both histone deacetylase (HDAC) activity and the growth of colon cancer cells. In this study, four identified phenolic compounds in peanut testae (resveratrol, p-coumaric acid, ferulic acid, and sinapinic acid) were investigated for their HDAC inhibitory and anticancer activities against colon cancer cell lines. In vitro study revealed that resveratrol exhibited the greatest HDAC inhibitory activity. Molecular docking studies demonstrated that all four compounds could bind both HDAC1 and HDAC2. Resveratrol exhibited the most effective antiproliferative activity against both human colon adenocarcinoma (HT29) and human colorectal carcinoma (HCT116) cells. Apoptosis induction by ferulic acid and resveratrol appeared to be associated with p53 activation in HCT116 cells. However, resveratrol, p-coumaric acid, ferulic acid, and sinapinic acid induced apoptosis of HT29 cells in a p53-independent manner. Low-concentration treatments of p-coumaric and ferulic acids resulted in cell cycle arrest of HCT116 cells. In contrast, high-concentration treatments of p-coumaric and ferulic acids showed cell death activation as evidenced by increased sub-G1 fractions. The induction of p21 by p-coumaric acid and resveratrol correlated well with the decreased CDK4 levels and cell cycle arrest. Resveratrol, p-coumaric acid, ferulic acid, and sinapinic acid caused activation of pERK1/2 in HCT116 cells, whereas ferulic and sinapinic acids caused downregulation of pERK1/2 in HT29 cells. These results suggest that these peanut phenolics may be potential antineoplastic agents for colon cancer chemoprevention/chemotherapy.

Key words: HDAC inhibitor, peanut (Arachis hypogaea L.), p-coumaric acid, ferulic acid, sinapinic acid, resveratrol

1. Introduction
Colorectal cancer, one of the most common cancers in developed countries, is closely linked with dietary and lifestyle practices (Jemal et al., 2010). Dietary intake of plant-derived phenolic-rich ingredients might be a good and feasible strategy for preventing and controlling the incidence of colorectal cancer (Center et al., 2009). Phenolic compounds are the most abundant antioxidants among the bioactive phytochemicals present in our diet (Edwards et al., 2010), serving as natural cancer chemopreventive agents (Link et al., 2010). Generally, the underlying mechanisms for their anticancer effects include antioxidant, antiproliferation, and the activation and/or inhibition of some subcellular signaling pathways, namely apoptosis and cell cycle progression (Yang et al., 2001). Among plant-based diets, peanut seeds (Arachis hypogaea L.) are popularly consumed worldwide and the peanut testa has been found to contain several types of phenolic compounds, including resveratrol, p-coumaric acid, ferulic acid, and sinapinic acid (Udenigwe et al., 2008; Khaopa et al., 2012). Peanut testae have been shown to possess potent antioxidant, anticancer, and antiinflammatory activities (Chang et al., 2006). We have previously demonstrated that phenolic-rich extracts of peanut testae exhibited histone deacetylase (HDAC) inhibitory and anticancer activities in several human cancer cells (Khaopa et al., 2015). However, the types of phenolic compounds conferring HDAC inhibitory and anticancer properties to peanut testa extracts remains to be investigated. Our previous study (Khaopa et al., 2015) demonstrated that peanut testa extracts possessing HDAC inhibitory activity could inhibit the growth of human colon adenocarcinoma (HT29) cells and human colorectal carcinoma (HCT116) cells. However, the cytotoxic effects of the phenolics found in peanut testa extracts on colon cancer cell lines HT29 (p53-mutant) and HCT116 (p53-wild type) have not yet been explored.

HDAC inhibitors have been shown to cause transcriptional activation of a few silenced tumor
suppressor genes by reversible epigenetic changes (Marks et al., 2000; Kim et al., 2013). At present, there remains a continuous need for exploring new HDAC inhibitors with improved pharmacological efficacy and unique modes of action. The loss of protein expression involved in growth inhibition and induction of apoptosis is one of the most common reasons for uncontrolled growth of cancer cells (Roberts et al., 2002). Tumor suppressor protein p53 plays a key role in preventing tumor cell growth (Banin et al., 1998), whereas cyclins and cyclin-dependent kinases (CDKs) regulate cell cycle progression. The induction of p21, a CDK inhibitor, is responsible for arresting cell cycle progression via p53-dependent and -independent pathways (Gartel, 2005). Decreased CDK4 expression and cell cycle progression via p53-mediated and -independent pathways (Gartel, 2005). Decreased CDK4 expression and increased p21WAF1 expression were associated with cell cycle arrest (Cho et al., 2001; Sherr et al., 2002). Moreover, a mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade is of great importance in regulating various cellular responses, including proliferation, differentiation, survival, and cell death. The cascade is activated through receptor-mediated signaling stimuli (Mordret, 1993). The ERK signaling is synonymous with cell proliferation and found to be deregulated in about one-third of all human cancers (Dhillon et al., 2007). In the ERK/MAPK model, ERK1/2 is activated upon phosphorylation by MEK1/2, which is itself activated when phosphorylated by Raf (Johnson et al., 1996). Cell death could be induced through ERK activation by the generation of reactive oxygen species (ROS) and endoplasmic reticulum stress predominantly via the PERK-eIF2α pathway (Arai et al., 2004). However, the mechanisms used by peanut phenolic compounds to inhibit cancer cell growth remain to be explored.

The investigation of HDAC inhibitory activity of phenolic compounds found in peanut seeds may lead to the discovery of safe and potentially chemopreventive natural agents. To validate that peanuts are a natural source of HDAC inhibitors, the HDAC inhibitory activity of peanut phenolic compounds, including resveratrol, p-coumaric acid, ferulic acid, and sinapinic acid, was demonstrated both in vitro and in cancer cell lines. The effects of these phenolic compounds on the proliferation, apoptosis, and cell cycle progression of colon cancer cells were demonstrated. In addition, the levels of p21, CDK4, p53, and pERK1/2 upon treatment with these phenolic compounds were also investigated.

2. Materials and methods

2.1. Materials

Propidium iodide, sodium butyrate (purity ≥98.5%), resveratrol (purity ≥99.0%), p-coumaric acid (purity ≥98.8%), ferulic acid (purity ≥99.0%), and sinapinic acid (purity ≥98.0%) were purchased from Sigma-Aldrich. Antibodies against H3, p21, CDK4, β-actin, pERK1/2, and ERK1/2 proteins were purchased from Cell Signaling Technology. Anti-p53 antibody was purchased from Santa Cruz Biotechnology. The Vybrant Apoptosis Assay Kit #2 and MTT were purchased from Molecular Probes, Invitrogen.

2.2. Cell culture

The colon cancer cell lines (HT29 and HCT116) and noncancer cell lines (Vero) used in this study were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) (GIBCO-BRL). The cells were incubated in a humidified incubator with an atmosphere of 5% CO2 at 37 °C.

2.3. In vitro HDAC inhibitory activity

In vitro HDAC inhibitory activity was determined using the Fluor-de-Lys HDAC Fluorometric Activity Assay Kit (Biomol). Four peanut phenolics, resveratrol, p-coumaric acid, ferulic acid, and sinapinic acid, at various concentrations, were assessed for their HDAC inhibitory activity against the mixture of HDAC enzymes from the HeLa nuclear extract provided with the kit. The assay was performed according to the manufacturer’s instructions. Fluorescence was measured using a Spectra Max Gemini XPS microplate spectrofluorometer (Molecular Devices) with excitation at 360 nm and emission at 460 nm.

2.4. In silico molecular docking studies

AutoDock tools were used to analyze the binding energy (ΔG) and inhibitory constant (Ki). The Molecular Graphics Laboratory tools and AutoDock 4.2 were downloaded from www.scripps.edu. Cygwin (for data storage) and Discovery Studio Visualizer 4.0 were downloaded from www.cygwin.com and www.accelerys.com, respectively. The chemical structure of the phenolic compounds was drawn in the form of a chemical diagram using Spartan Student 4.1.1. The human HDAC1 and HDAC2 crystal structures were collected from a databank (http://www.rcsb.org/pdb/staticHelp.do?p=help/ligandSearch.html). For each ligand, the 10 best positions were generated and scored using the AutoDock 4.2 scoring functions according to methods previously described (Park et al., 2006). The conformations with the best docking, as determined from the clustering histogram, were those with low binding energy. Binding energy is a measure of the affinity of a ligand–protein complex, or the difference between the energy of the complex and the sum of the energies of each molecule separately. The binding energy of the individual compound was calculated using the following formula:

\[
\text{binding energy} = A + B + C - D,
\]

where A denotes the final intermolecular energy + Van der Waals energy + hydrogen bonds + desolvation energy + electrostatic energy (kcal/mol), B denotes the final total internal energy (kcal/mol), C denotes the torsional free internal energy (kcal/mol).
Cells were seeded into a 5.5-cm dish (~1 × 10⁶ cells) as previously described by Poolman and Brooks (1998). Cell cycle phase distribution was evaluated using a method described by temperature and analyzed by flow cytometry.

The samples were then kept in the dark for 15 min at room temperature. After 2 h of incubation, formazan produced in the culture cells was dissolved with DMSO, and the absorbance at 550 nm was measured with a microtiter plate reader (Bio-Rad Laboratories) using 655 nm as a reference wavelength.

2.6. Cell cycle analysis by flow cytometry

Cells were harvested, centrifuged, and washed twice with ice-cold PBS. Cells were suspended in 100 µL ice-cold annexin-binding buffer and stained with Alexa Fluor 488-annexin V and propidium iodide (PI) solutions. The samples were then kept in the dark for 15 min at room temperature and analyzed by flow cytometry.

2.7. Apoptosis analysis by flow cytometry

Induction of apoptosis was assayed using the Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Invitrogen) according to the manufacturer's instructions with a BD FACSCanto II flow cytometer (Becton Dickinson). To start, cells (~1 × 10⁶) were seeded into a 5.5-cm dish and incubated for 24 h. The cells were then treated with three different concentrations of resveratrol (50, 100, and 150 µM), p-coumaric acid (1, 2.5, and 5 mM), ferulic acid (1, 2.5, and 5 mM), and sinapinic acid (1, 2.5, and 5 mM) or solvent (DMSO, 0.37%) for 24 h. After incubation, the treated cells were harvested, centrifuged, and washed twice with ice-cold PBS. Cells were suspended in 100 µL ice-cold annexin-binding buffer and stained with Alexa Fluor 488-annexin V and propidium iodide (PI) solutions. The samples were then kept in the dark for 15 min at room temperature and analyzed by flow cytometry.

2.8. Western blot analysis

Levels of the proteins of interest were detected by western blot following the established method. The total cellular proteins were prepared from drug-treated and solvent-treated cells using RIPA lysis buffer. Equal amounts of protein (30 µg) were separated by 10%–12.5% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked with 5% skim milk for 1 h and subsequently incubated overnight with a primary antibody: anti-p21, antiacetylated H3, anti-CDK4, anti-ERK1/2, anti-ERK1/2, anti-p53, or anti-β-actin. The proteins were then probed with a horseradish peroxidase-conjugated secondary antibody at room temperature. Protein bands were visualized using ECL prime chemiluminescence (Amersham).

2.9 Statistical analysis

Data are expressed as the mean ± standard deviation (SD) from three independent experiments. Duncan's post hoc test was carried out using SPSS 20.0 (IBM Corporation). Significant differences (P < 0.05) among phenolic compounds were analyzed using one-way analysis of variance.

3. Results

3.1. HDAC inhibitory activity of four peanut phenolic compounds

The HDAC inhibitory activity of four peanut phenolic compounds (Udenigwe et al., 2008; Khaopha et al., 2012) was investigated both in vitro and in silico in comparison with a well-known HDAC inhibitor, sodium butyrate (NaB). As shown in Table 1, all four phenolic compounds could inhibit HDAC activity in vitro and resveratrol had the lowest IC₅₀ value (0.4 ± 0.05 mM) against the crude HDAC enzymes from the HeLa nuclear extract, followed by ferulic acid (1.9 ± 0.1 mM), sinapinic acid (2.3 ± 0.1 mM), and p-coumaric acid (2.6 ± 0.1 mM). Compared to the IC₅₀ value of NaB (1.02 ± 0.1 mM), only resveratrol possessed more potent HDAC inhibitory activity than NaB in vitro. To support the in vitro results, an in silico study or molecular docking was conducted using these compounds as ligand molecules docking with crystal structures of both human HDAC1 and HDAC2, the two major HDAC family members found in most transcriptional repression complexes (Hassig et al., 1998). The results revealed that all of the inhibitor compounds bind with one or more amino acids in the active pockets of the enzymes (data not shown). The calculated binding energy (ΔG) and inhibitory constant (Ki) of the individual compounds are shown in Table 1. Theoretically, all four phenolic compounds showed very good binding energy compared to that of the well-known HDAC inhibitor NaB. According to the binding energy and inhibitory constant, all four phenolic compounds (ΔG = –3.4 to –4.9 kcal/mol, Ki =
0.3 to 3.3 mM) appeared to interact with HDAC1 more efficiently than NaB ($\Delta G = -2.9$ kcal/mol, $K_i = 8.0$ mM). In contrast, only $p$-coumaric acid ($\Delta G = -5.1$ kcal/mol, $K_i = 170.8$ µM) showed a better interaction with HDAC2 than NaB ($\Delta G = -4.7$ kcal/mol, $K_i = 375.3$ µM). These results indicate that all four phenolic compounds found in peanut testa extracts (Khaopha et al., 2015) function as HDAC inhibitors capable of inhibiting HDAC enzymes.

### 3.2. Antiproliferative activity of four peanut phenolic compounds

To determine the antiproliferative activity of these compounds, the MTT assay was used to evaluate their growth inhibition activity against human colon cancer cell lines HT29 and HCT116 and noncancer (Vero) cells. A dose-response curve showed that all four phenolic compounds inhibited the proliferation of colon cancer cell lines in a time-dependent and dose-dependent manner (data not shown). Cellular sensitivity was determined by calculating the IC$_{50}$ against each compound (Table 2). Resveratrol produced a more profound cytotoxic effect in colon cancer cells. Consistent with our previous report (Senawong et al., 2014), $p$-coumaric, ferulic, and sinapinic acids exhibited similar IC$_{50}$ values against HT29, HCT116, and Vero cells at 72 h of exposure. In line with our findings, previous reports documented the growth inhibition effects of plant phenolics on colon cancer cells (Yi et al., 2005). In addition, $p$-coumaric, ferulic, and sinapinic acids were more cytotoxic than NaB. However, it has been reported that sinapinic acid inhibited the growth of HeLa and Jurkat cells less effectively than sodium butyrate (Senawong et al., 2013).

### 3.3. Hyperacetylation of histone H3 by peanut phenolic compounds in colon cancer cells

As presented above, we observed that all four phenolic compounds possessed in vitro HDAC inhibitory activity and inhibited the growth of both colon cancer cell lines. Therefore, we aimed to explore the HDAC inhibitory activity of these compounds in both cancer cell lines by determining the acetylation status of histone H3 using western blot analysis. As demonstrated in Figure 1, all four phenolic compounds, $p$-coumaric acid (Figures 1A and 1B), ferulic acid (Figures 1C and 1D), sinapinic acid (Figures 1E and 1F), and resveratrol (Figures 1G and 1H), caused hyperacetylation of histone H3 in both HT29 and

**Table 1.** Half maximal inhibitory concentrations (IC$_{50}$) of phenolic compounds after exposure to HDAC enzymes in vitro and their binding energy and inhibitory constants against HDAC1 and HDAC2 in silico.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Chemical structure</th>
<th>In vitro</th>
<th>In silico</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDACs$^a$</td>
<td>HDAC1</td>
<td>HDAC2</td>
</tr>
<tr>
<td></td>
<td>IC$_{50}$ values$^b$ (mM)</td>
<td>Binding energy ($\Delta G$) (kcal/mol)</td>
<td>Inhibitory constant ($K_i$)</td>
</tr>
<tr>
<td>$p$-Coumaric acid</td>
<td>2.6 ± 0.1</td>
<td>-3.5</td>
<td>2.9 mM</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1.9 ± 0.1</td>
<td>-4.3</td>
<td>673.3 µM</td>
</tr>
<tr>
<td>Sinapinic acid</td>
<td>2.3 ± 0.1</td>
<td>-3.4</td>
<td>3.3 mM</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.4 ± 0.1</td>
<td>-4.9</td>
<td>271.6 µM</td>
</tr>
<tr>
<td>Sodium butyrate$^c$</td>
<td>1.0 ± 0.1</td>
<td>-2.9</td>
<td>8.0 mM</td>
</tr>
</tbody>
</table>

$^a$The mixture of HDAC enzymes in the HeLa nuclear extract.

$^b$IC$_{50}$ values are shown as mean ± SD from three independent experiments, representing the concentrations of the indicated compounds that inhibit 50% of HDAC activity.

$^c$A well-known HDAC inhibitor.
HCT116 cells, indicating that all four compounds could inhibit HDAC activity in a cellular context compared with the HDAC inhibitor NaB (Figures 1I and 1J). Among the four compounds, resveratrol was shown to possess the most potent HDAC inhibitory activity in both HT29 (Figure 1G) and HCT116 (Figure 1H) cells, which correlates well with its activity in vitro (Table 1).

3.4 Peanut phenolic compounds induce apoptosis in human colon cancer cell lines

To elucidate the mechanism underlying cell growth inhibition by the peanut phenolic compounds, induction of apoptosis in colon cancer cell lines was examined. As shown in Figure 2, all four phenolic compounds exhibited a significant effect on induction of apoptosis in both HT29 and HCT116 cells. In the HT29 cells, treatments with increasing concentrations of p-coumaric acid (Figure 2A), ferulic acid (Figure 2B), sinapinic acid (Figure 2C), resveratrol (Figure 2D), and NaB (Figure 2E) increased the percentage of apoptotic cells. Similarly, the percentage of HCT116 apoptotic cells also increased after treatment with these phenolic compounds (Figures 2F–2I) and NaB (Figure 2J). In addition, the percentage of apoptotic noncancer cells was lower than that of apoptotic cancerous cells, indicating that the peanut phenolic compounds (Figures 2K–2N) were less toxic to a noncancer cell line than to cancerous cell lines. In contrast, NaB exhibited more toxicity to a noncancer cell line (Figure 2O) than to cancerous cell lines (Figures 2E and 2J).

3.5. Peanut phenolic compounds induce cell cycle arrest in human colon cancer cell lines

Cell cycle arrest was further examined by flow cytometry after treatment with the phenolic compounds. As presented in Table 3, the HT29 cells showed a significant increase in the number of sub-G1 cells along with concomitant decrease of the G0/G1 population when treated with resveratrol, p-coumaric acid, ferulic acid, and sinapinic acid. NaB caused an increase of cells in the S phase but a decrease in the G2/M phase. In contrast, the HCT116 cells were arrested in the G0/G1 phase after treatment with p-coumaric acid, ferulic acid, and sinapinic acid, whereas an S phase arrest was observed with the resveratrol treatment. Of note, the NaB-treated HCT116 cells showed a prominent sub-G1 fraction.

3.6. Peanut phenolic compounds induced inhibition of ERK1/2 pathway

The effects of the phenolic compounds on the MAPK (ERK) signaling pathway of colon cancer cells were investigated by western blot analysis. p-Coumaric acid caused an increase in the level of pERK1/2 in both the HT29 (Figure 3A) and the HCT116 (Figure 3B) cells. In contrast, ferulic acid caused an inhibition of the MAPK signaling pathway in the HT29 cells (Figure 3C), but activated the MAPK signaling pathway in the HCT116 cells (Figure 3D). Similarly, sinapinic acid decreased the pERK1/2 level in the HT29 cells (Figure 3E), but increased the pERK1/2 level in the HCT116 cells (Figure 3F). Resveratrol increased the level of pERK1/2 at all concentrations tested in the HT29 cells (Figure 3G), and at the highest concentration (200 µM) in the HCT116 cells (Figure 3H). Furthermore, the NaB treatments caused a reduction of pERK1/2 in both the HT29 (Figure 3I) and the HCT116 (Figure 3J) cells, especially at high concentration treatments.

3.7. Western blot analysis of cell cycle and apoptosis regulatory proteins

To explore the molecular mechanisms underlying growth inhibition of colon cancer cells, HT29 (p53-mutant) and HCT116 (p53-wild type), by peanut phenolic compounds, the expression of some cell cycle and apoptosis regulatory proteins was examined. As shown in Table 4, all four phenolic compounds downregulated p53 and increased p21 expression in both HT29 and HCT116 cells. In addition, resveratrol caused an increase in the expression of p27 in both cell lines, whereas NaB decreased the expression of p27 in the HT29 cells but increased it in the HCT116 cells. The downregulation of p53 and the upregulation of p21 and p27 were especially prominent in the NaB-treated HCT116 cells (Figure 4C). Furthermore, the NaB treatments increased the expression of Bax and decreased the expression of Bcl-2 in both cell lines, indicating that NaB caused a pro-apoptotic effect on both cell lines (Figures 4D and 4E). In contrast, resveratrol increased the expression of Bcl-2 in both cell lines and decreased the expression of Bax in the HT29 cells but increased it in the HCT116 cells (Figures 4F and 4G). These results suggest that the peanut phenolic compounds induce apoptosis through a p53-dependent pathway, whereas NaB has a more pro-apoptotic effect on both cell lines.

Table 2. Antiproliferative activity of the four phenolic compounds represented by IC₅₀ values after exposures to colon cancer cell lines for 24, 48, and 72 h.

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>IC₅₀ values a</th>
<th>HT29 cells</th>
<th>HCT116 cells</th>
<th>Vero cells b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>72 h</td>
<td>24 h</td>
</tr>
<tr>
<td>p-Coumaric acid (mM)</td>
<td>&gt;3</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>&gt;3</td>
</tr>
<tr>
<td>Ferulic acid (mM)</td>
<td>&gt;3</td>
<td>2.4 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>&gt;3</td>
</tr>
<tr>
<td>Sinapinic acid (mM)</td>
<td>&gt;3</td>
<td>2.7 ± 0.3</td>
<td>2.6 ± 0.4</td>
<td>&gt;3</td>
</tr>
<tr>
<td>Resveratrol (µM)</td>
<td>&gt;200</td>
<td>131.2 ± 9.8</td>
<td>99.8 ± 8.7</td>
<td>190.3 ± 1.2</td>
</tr>
<tr>
<td>Sodium butyrate c (mM)</td>
<td>&gt;6</td>
<td>5.4 ± 0.7</td>
<td>3.7 ± 0.2</td>
<td>&gt;6</td>
</tr>
</tbody>
</table>

aIC₅₀ values or half maximal inhibitory concentrations are shown as means ± SD from three independent experiments.
bNoncancer cell line.
cA well-known HDAC inhibitor.

HCT116 cells, indicating that all four compounds could inhibit HDAC activity in a cellular context compared with the HDAC inhibitor NaB (Figures 1I and 1J). Among the four compounds, resveratrol was shown to possess the most potent HDAC inhibitory activity in both HT29 (Figure 1G) and HCT116 (Figure 1H) cells, which correlates well with its activity in vitro (Table 1).
Figure 1. Hyperacetylation of histone H3 by peanut phenolic compounds and NaB. Acetylated histone-H3 levels increased in HT29 (A, C, E, G, and I) and HCT116 (B, D, F, H, and J) cells after exposure to varying concentrations of phenolic compounds for 24 h. The control (C) represents the level of acetylated (Ac)–H3 in the solvent control treatment. β-actin was used as a loading control. Quantification of the relative expression of Ac-H3 in HT29 and HCT116 cells was determined using Genetool software (Genesys).
**Figure 2.** The effect of peanut phenolic compounds on apoptosis induction. HT29, HCT116, and noncancer (Vero) cells were incubated for 24 h with $p$-coumaric acid (A, F, and K), ferulic acid (B, G, and L), sinapinic acid (C, H, and M), resveratrol (D, I, and N), and the well-known HDAC inhibitor NaB (E, J, and O). S and P represent solvent (DMSO) and camptothecin treatments, respectively. *: Significant difference between solvent control and treatment ($P < 0.05$).
Table 3: Analysis of the cell cycle distribution of the colon cancer cell lines treated with different phenolic compounds for 24 h.

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Control</th>
<th>p-Coumaric acid (mM)</th>
<th>Ferulic acid (mM)</th>
<th>NaB (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29 cells</td>
<td>Sub-G1</td>
<td>1.0</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
<td>7.6 ± 2.4</td>
<td>15.3 ± 4.3</td>
<td>20.6 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>44.4 ± 1.6</td>
<td>28.4 ± 1.3</td>
<td>35.0 ± 10.1</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>16 ± 1.7</td>
<td>18.5 ± 1.0</td>
<td>30.0 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>91 ± 3.1</td>
<td>184.5 ± 5.1</td>
<td>200.0 ± 9.3</td>
</tr>
<tr>
<td>Cell cycle phase</td>
<td>HT-29 cells</td>
<td>1.0</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Sub-G1</td>
<td>4.4 ± 0.3</td>
<td>5.5 ± 0.5</td>
<td>11.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
<td>45.1 ± 1.6</td>
<td>28.4 ± 1.3</td>
<td>35.0 ± 9.9</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>151 ± 6.3</td>
<td>199.2 ± 4.9</td>
<td>31.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>197 ± 2.4</td>
<td>251 ± 4.3</td>
<td>311 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>197 ± 2.4</td>
<td>251 ± 4.3</td>
<td>311 ± 7.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Control</th>
<th>p-Coumaric acid (mM)</th>
<th>Ferulic acid (mM)</th>
<th>NaB (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116 cells</td>
<td>Sub-G1</td>
<td>1.9 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
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<tr>
<td></td>
<td>G2/M</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
<td>17 ± 0.2</td>
</tr>
</tbody>
</table>

Cell numbers are presented as a percentage of the total analyzed cells and the values are shown as mean ± SD from three independent experiments. Cells were treated with a solvent.
Figure 3. The effect of peanut phenolic compounds on ERK1/2 activation. HT29 and HCT116 cells were exposed to 0.37% DMSO (solvent control) and varied concentrations of p-coumaric acid (A and B), ferulic acid (C and D), sinapinic acid (E and F), resveratrol (G and H), and NaB (I and J) for 4 h. Serum-starved HT29 and HCT116 cells were stimulated with 750 ng/mL EGF for 1 h after 3 h of preincubation with or without phenolic compounds. Total ERK1/2 was used as a loading control. Quantification of the relative expression of pERK1/2 in HT29 and HCT116 cells was determined using Genetool software (Genesys).
proteins were evaluated by western blotting. In this study, three proteins related to cell growth inhibition (p21, CDK4, and p53) were investigated. p21 is one of the p53-regulated protein targets of HDAC inhibitor-mediated suppression to control cell cycle progression. In the HT29 cells, p-coumaric acid (Figure 4A) induced p21 expression, whereas ferulic acid (Figure 4B) and sinapinic acid (Figure 4C) acids decreased p21 level. Resveratrol (Figure 4D) and NaB (Figure 4E) also increased the p21 level compared to the solvent control treatment (Figure 4F). CDK4, a protein associated with cell cycle regulation, was also detected in the same condition. In the HT29 cells, all of the phenolic compounds and NaB caused a downregulation of CDK4 (Figure 4G). Mutant p53 protein expression was constant for the phenolic compounds and NaB treatments (Figure 4H) in the HT29 cells. In HCT116 cells, p21 expression was increased when treated with p-coumaric acid (Figure 4I), but it decreased when cells were treated with ferulic acid (Figure 4J) and sinapinic acid (Figure 4K) acids. Furthermore, resveratrol (Figure 4L) at a concentration of 150 µM caused a decrease of p21 in the HCT116 cells. The increase of p21 upon treatments with p-coumaric acid and NaB (Figure 4M) appeared to be independent of p53 in the HCT116 cells (Figure 4N). All four phenolic compounds and NaB caused a reduction in CDK4 expression in the HCT116 cells (Figure 4O). In the HCT116 cells, the expression of p53 remained unaltered after the p-coumaric acid and sinapinic acid treatments, but it was upregulated when treated with ferulic acid and resveratrol (Figure 4P). However, the p53 level seemed to decrease after NaB treatment (Figure 4P). Interestingly, the resveratrol-treated HCT116 cells exhibited an increased p53 level (Figure 4P) but unaltered or decreased p21 levels (Figure 4N), suggesting that the increase of p53 level in this cell line may not cause G0/G1 cell cycle arrest. In this context, the role of p21 is particularly complicated, as this protein can be activated by p53-independent mechanisms upon exposure to p-coumaric acid and NaB (Figure 4N).

4. Discussion
The principal aim of cancer chemoprevention is the use of pharmacological or natural agents to prevent or stop the process of tumorigenesis. Here we demonstrated that all four phenolic compounds found in peanut testa extracts (Khaopha et al., 2015) possess HDAC inhibitory activity and may inhibit the growth of colon cancer cells. Among the four phenolic compounds tested, resveratrol exhibited the most effective HDAC inhibition (Table 1; Figure 1) and cell growth inhibition (Table 2). An imbalance between acetylation and deacetylation changes the structure and the activity of histone proteins, influencing cancer cell functions such as gene expression, cell cycle progression, and cell death pathways (Drummond et al., 2005; Peart et al., 2005). In addition, HDAC inhibitor-induced accumulation of acetylated histones may affect cell cycle progression by altering the ability of tumor cells to undergo mitosis (Warrener et al., 2003). However, the relationship between hyperacetylation of histone proteins and cell growth inhibition is still unclear. Previous studies demonstrated that combination treatments of HDAC inhibitors and the current anticancer drug cisplatin may lead to enhanced anticancer efficacy and reduced cytotoxicity to normal cells (Shen et al., 2007; Ong et al., 2012; Asgar et al., 2016). Drug combination studies between the peanut phenolic-based HDAC inhibitors and current anticancer drugs such as cisplatin and 5-fluorouracil are of future interest with the aim to reduce toxicity and resistance based on their differences in drug targets. Consistent with our results, resveratrol has been found to inhibit proliferation in a number of cancer cell lines (Agrawal et al., 2002). Moreover, resveratrol as well as some other phenolic compounds of natural origin, including curcumin and capsaicin, exhibited growth inhibition against HL-60, K-562, MCF-7, and HeLa cells (Roy et al., 2002). It is noteworthy that all four phenolic compounds in this study were less toxic on noncancer cells than cancer cells, consistent with many other phenolic compounds (Nakajima et al., 2009). Similarly, the noncancer cell line appeared to be resistant to both sodium butyrate and phenolic-rich extracts of Hydrophytum formicarum Jack rhizome (Senawong et al., 2013).

According to the apoptosis results, it is evident that apoptosis induction is the underlying mechanism for cancer cell growth inhibition by the four peanut phenolic compounds (Figure 2). Regarding phenolic compound-induced apoptosis, phenolic-rich extracts and sinapinic acid from H. formicarum also inhibited the growth of HeLa cells by induction of apoptosis (Senawong et al., 2013). Similarly, the natural phenolic compounds curcumin, resveratrol, and capsaicin act as chemopreventive agents by inducing apoptosis in tumor cells (Roy et al., 2002). Phenolic compounds from blueberries also showed growth inhibition and apoptosis induction of colon cancer cell lines (Yi et al., 2005). Resveratrol induced a decrease in proliferation rates and an increase in apoptosis in prostate cancer cell lines in a dose-dependent manner (Benitez et al., 2007). In addition, p-coumaric and ferulic acids have previously been reported to induce cancer cell apoptosis (Jaganathan et al., 2013; Peng et al., 2013).

The cell cycle profiles indicate that all four phenolic compounds inhibited cancer cell growth via induction of not only apoptosis but also cell cycle arrest (Table 3). Moreover, a noncancer cell line (Vero cells) was not arrested at any particular phase of the cell cycle (data not shown). All four phenolic compounds and NaB induced a small increase in sub-G1 in the Vero cells. Our findings
Resveratrol was also reported to induce growth inhibition along with arrest in the S phase of several human cancer cell lines (Joe et al., 2002).

Treatment of the HCT116 cells with all four phenolic compounds resulted in ERK1/2 activations (Figures 3B, 3D, 3F, and 3H). The observed ERK1/2 activations may be associated with oxidative stress upon drug treatment.

on cell cycle arrest are in agreement with several findings on induction of cancer cell cycle arrest by some phenolic compounds. For example, the growth inhibitory activity of ellagic acid was accompanied by induction of arrest of the cell cycle in the G0/G1 phase of human bladder cancer T24 cells (Li et al., 2005). Resveratrol was also reported to...

Figure 4. The effect of peanut phenolic compounds on the expression levels of cell cycle and apoptosis regulatory proteins. The protein expression levels of p21, CDK4, and p53 in HT29 (A–H) and HCT116 (I–P) cells were determined after treatment with phenolic compounds and NaB where β-actin was used as a loading control. Proteins were extracted from cell lysates, separated by SDS-PAGE, and probed with the respective antibodies. C represents the protein level in the solvent control treatment. Quantification of the relative expression of these proteins in HT29 (F–H) and HCT116 (N–P) cells was determined using Genetool software (Genesys).
Previous reports have revealed that ERK1/2 activation induced ROS generation and endoplasmic reticulum stress mainly via the PERK-eIF2α pathway, causing apoptosis (Tan and Chiu, 2013). The activation of MAPKs through intracellular ROS has been documented (Martindale and Holbrook, 2002). Similarly, intracellular ROS leads to activation of ERK1/2 and induce apoptosis in cancer cells (Shin et al., 2009). The precise role of ROS in the signaling of apoptosis is very complicated, as both the intrinsic and extrinsic pathways are known to be associated with ROS (Mates et al., 2008). Previous reports demonstrated that oxidative stress induced by chemopreventive drugs reduced mitochondrial membrane potential and caused cytochrome c to be released from the mitochondria, resulting in the activation of caspase-9 (Bagriacik et al., 2007). However, our findings suggest a more complicated role of ERK1/2 inhibition in promoting cell cycle arrest and apoptosis upon phenolic compound treatment.

It is widely accepted that p53 expression regulates apoptosis and/or cell cycle arrest in response to DNA damage by anticancer agents. In accordance with previous reports that HDAC inhibitors caused cell death in a p53-dependent manner (Zhao et al., 2006), ferulic acid and resveratrol could induce cell death and activate p53 expression in HCT116 cells (Figure 4P). However, both upregulation and downregulation of p21 were observed upon treatment of the colon cancer cells with the four phenolic compounds (Figures 4F and 4N). This suggests that modes of cell death other than apoptosis may operate in tumor cells following exposure to sinapinic acid, or, more generally, to DNA-damaging agents (te Poele et al., 2002). Expression of p21 coincides with the hyperacetylation of histones H3 and H4 in its promoter region (Richon et al., 2002), indicating that HDAC enzymes negatively regulate p21 expression in many cell types. However, in HT29 cells, p53-independent induction of p21 expression seems to be a prerequisite for apoptosis (Agrawal et al., 2002) and also seems to sensitize tumor cells to the actions of different agents. Interestingly, our observations do not comply with either view. In fact, while the induction of p21 expression in p53-proficient and -deficient cell lines was responsive to drug treatment, this did not parallel the difference in apoptotic cells between the HCT116 and HT29 cells. Regarding CDK4, all phenolic compounds tested in this study caused downregulation of CDK4 in the HCT116 cells (Figure 4O). As with NaB treatment (Sherr, 1994), the decreased CDK4 level resulting from the phenolic compound treatments may facilitate controlling the cell cycle progression at the G1 phase in HCT116 cells.

Similar to our observation, it has been shown that resveratrol induced the expression of p21/WAF1 and decreased CDK4 expression in human vascular smooth muscle cells in which p21 expression was p53-independent (Lee and Moon, 2005). However, resveratrol induced-cell cycle arrest was mediated by increased expression of the modulator proteins p53 and p21 and decreased CDK4 expression in LNCaP cells (Benitez et al., 2007). Some research findings with regard to p21 and CDK4 coincide with our observations only for p-coumaric acid and NaB treatments. Ellagic acid increased p53 and p21 but decreased CDK2 gene expression, which can lead to the G0/G1 arrest of T24 cells (Li et al., 2005). It was reported that activation of p53/p21(WAF1/CIP1) expression played a role in G1 arrest and apoptosis in cancer cells treated with ellagic acid (Narayanan et al., 1999).

The four peanut phenolic compounds exhibited antiproliferative activity against colon cancer cell lines, which was accompanied by apoptosis with p53 either dependently or independently. As these compounds also exhibited HDAC inhibitory activity, one may envision that inhibition of HDAC activity may lead to a decreased expression of CDK4 but increased expression of p21, which eventually could block the cell cycle progression. An oncogenic cell signaling process involving pERK1/2 activation was also responsible for growth inhibition of HCT116 cells. This study has shown that peanut phenolic compounds possessing HDAC inhibitory activity could induce apoptosis and cell cycle arrest in tumor cells, which could have an important implication in the further development of cancer chemopreventive agents.

In summary, a possible mechanism for the protective effect of peanut phenolic compounds against colon cancer relates to their antiproliferative and proapoptotic effects, leading to downregulation of cell cycle proteins and an increase in the apoptosis of colon cancer cells.

Acknowledgments
This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Food and Functional Food Research Cluster of Khon Kaen University. Grateful acknowledgment is also made to the Thailand Research Fund, the Commission of Higher Education, and Khon Kaen University for providing financial support to this research through a Distinguished Research Professor Grant to Dr Aran Patanothai.
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