Phenolic Extracts from Insoluble-Bound Fraction of Whole Wheat Inhibit the Proliferation of Colon Cancer Cells

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Abstract
The insoluble-bound fraction of two commercial blends of whole and refined wheat (Barretta and Magnolia) was assessed for its total phenolic content, phenolic acid composition, antiproliferative activity, and cytotoxic effects towards Caco-2 human colon cancer cells. The total phenolic content of the insoluble-bound fraction of Barretta and Magnolia whole wheat was 97.5 and 95.8 mg gallic acid equivalents/100 g whole wheat, respectively. The total phenolic content of the insoluble-bound fraction of Barretta and Magnolia refined wheat was 13.8 and 12.8 mg gallic acid equivalents/100 g. Ferulic acid was the predominant phenolic acid found in both commercial blends of whole and refined wheat. p-Coumaric acid and caffeic acid were also detected in the insoluble-bound fraction of whole wheat. However, no caffeic acid was detected in the insoluble-bound fraction of refined wheat. Phenolic extracts (100 mg/mL) from the insoluble-bound fraction of whole wheat were able to significantly (p < 0.05) reduce the number of Caco-2 cells at 24 and 96 hours post-treatment. Phenolic extracts from the insoluble-bound fraction of refined wheat did not have similar effects. The reduction in cell number was not associated with an increase in cell death. These data suggest that phenolic extracts from the insoluble-bound fraction of whole wheat but not refined wheat inhibit the proliferation of human colon cancer cells, in vitro. These data also support several epidemiological studies linking increased whole grain consumption and reduced risk of colorectal cancer.

Keywords: Whole wheat; refined wheat; insoluble-bound phenolics; phenolic acids; Caco-2 cell; colon cancer; diet and cancer.

1. Introduction

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis [1]. Milling, the separation of the germ and bran from the endosperm so that the endosperm (refined grain) can be ground into flour, is the first processing step in the manufacturing of food products. Most of the grain products consumed in the US are consumed as refined grain food products [2]. Because of the proportion of which refined grain food products are consumed in the US and the potential health benefit of whole grain food product consumption, it is important to determine the potential health benefit of both whole and refined grain food product consumption.

Phytochemicals are non-nutrient, naturally occurring plant compounds found in fruits, vegetables, and grains. Grain processing may influence the amounts of phytochemicals found in grains [3]. The grain phytochemicals of particular interest due to their potential health benefits are phenolics; compounds with one or more aromatic ring and one or more hydroxyl group. The predominant phenolic compounds found in whole grains are phenolic acids (Figure 1). Phenolic acids, hydroxybenzoic-acid and hydroxycinnamic-acid derivatives, are generally found esterified or bound to cell wall polymers and are therefore insoluble when solvents are used to extract phenolic compounds from whole or refined grains. These compounds can be released from the cell wall by the enzymatic activity of gut microflora. Andreasen et al. proposed a mechanism by which insoluble-bound phenolic compounds may be released in the lower gastrointestinal tract by gut microflora [4] where they may exhibit their potential health benefits [1]. The potential health benefits of phenolic acids may be due to their ability to inhibit the proliferation of human cancer cells [5-7].

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**Figure 1**: Structure of phenolic acids.

<table>
<thead>
<tr>
<th>a. Hydroxycinnamic acids</th>
<th>b. Hydroxybenzoic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic acid</td>
<td>p-Hydroxybenzoic Acid</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>Syringic Acid</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Vanillic Acid</td>
</tr>
</tbody>
</table>

Phenolic acids from the insoluble-bound fraction of whole and refined grains may also have cytotoxicity, the potential of a compound or other treatment to induce cell death [8]. Cell death can be due to necrosis, an increase in cellular volume resulting in rupture of the cell, or apoptosis, the reduction of cellular volume finally resulting in plasma membrane blebbing, amongst other causes [9]. Upon cell death, the integrity of the cell membrane is compromised, causing the cell to leak its contents into the culture medium. Lactate dehydrogenase (LDH) activity in the culture medium can then be assessed as a marker of cytotoxicity and is commonly used to assess cytotoxicity in a wide range of cell lines in vitro [10, 11].

Mattila et al. (2005) reported the phenolic acid content of whole and refined wheat [12]. Adom et al. (2005) reported the total phenolic content and ferulic acid content of refined wheat [13]. Though the two previous studies reported the phenolic content and composition of refined wheat, the total phenolic content and phenolic composition of the insoluble-bound fraction of refined wheat was not reported.

It is important to determine the total phenolic content, phenolic acid content, and antiproliferative effects of the insoluble-bound fraction of refined wheat, as many of the food products consumed are made from refined wheat. The objectives of this study were to determine the total phenolic content and phenolic acid composition of whole and refined wheat, determine the cytotoxic and antiproliferative effects of phenolic acids in Caco-2 colon cancer cells, and determine the cytotoxic and antiproliferative effects of phenolic extracts from the insoluble-bound fraction of whole and refined wheat in Caco-2 colon cancer cells.

**2. Methods**

**2.1. Samples**

Two commercial blends of soft red wheat (Barretta and Magnolia) and their refined flours (refined wheat) were obtained from Kraft Foods (East Hanover, NJ). The whole seed (whole wheat) was milled to a fine powder using a 20, 40, and 60 mesh size screen successively and mixed thoroughly. All samples were stored at -20°C until extraction.

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2.2. Extraction of insoluble-bound phenolics

Insoluble-bound phenolics were extracted from the residue resulting from free phenolic extraction [14]. The residue was first digested with 2 M sodium hydroxide at room temperature for 1 h whilst shaken under nitrogen. The mixture was then neutralized with concentrated hydrochloric acid. Hexanes were used to extract lipids from the mixture. Phenolics from the remaining mixture were then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness. The phenolics were reconstituted in methanol:hydrochloric acid (1 M, 85:15 v/v) for the determination of total phenolic content and phenolic acid content or reconstituted in deionized water for use in cell culture experiments. All samples were stored at -40°C until analysis.

2.3. Determination of total phenolic content

Total phenolic content was determined using the previously described Folin-Ciocalteu reagent colorimetric method [14, 15]. Briefly, phenolics extracts were reacted with Folin-Ciocalteu Reagent and then neutralized with sodium carbonate. After 90 minutes, the absorbance of the resulting solution was measured at 760 nm. Gallic acid was used as the standard and total phenolic content was expressed as mg GAE/100 g sample.

2.4. Determination of phenolic acid composition

Phenolic acid composition was determined using a previously reported and validated rp-HPLC-DAD method [14]. Samples were filtered through a 0.45 µm filter prior to analysis. Briefly, the mobile phase [(A) water at pH 2.8 with acetic acid and (B) acetonitrile:water 70:30 v/v to pH 2.8 with acetic acid] was delivered using a Waters 600E quaternary pump at a flow of 1.5 mL/min using the following gradient: 0 – 10% B for 2.5 minutes, 10 – 12% B for 3.5 minutes, 12 – 23% B for 10 minutes, 23 – 95% B for 4 minutes. Seventy five microliters of sample were injected using a Water 717 Autosampler. Separation of phenolic compounds was done using a C18 column (5 µm, 250 mm x 4.6 mm column; Grace Vydac, Baltimore, MD). Phenolic acids were detected using a Waters 996 Photodiode Array Detector. Each injection was monitored at 282 nm. Data signals were acquired and processed using Waters Empower software (2002) (Waters Corp., Milford, MA).

2.5. Cell culture

Caco-2 cells (colorectal adenocarcinoma) were grown in growth medium (DMEM supplemented with 5% FBS, 10 mM HEPES Buffer to pH 7.4, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin) and were maintained at 37°C and 5% CO₂ as described previously [16].

2.6. Cell counting assay

The effects of phenolic acids and insoluble-bound phenolics extracts from whole and refined wheat on the number of Caco-2 cells were assessed using the previously described methylene blue stain cell counting assay [17] with modification that allowed for cell counting in a 96-well plate [16]. Caco-2 cells were plated at a density of 4 x 10⁴ and 2.5 x 10⁵ cells per well to assess cell number at 24 and 96 hours post-treatment, respectively. The growth medium was then replaced by growth medium containing phenolic acids (2% v/v DMSO in growth medium) or phenolics extracts from the insoluble-bound fraction of whole or refined wheat (10% water in growth medium). The cells were incubated at 37°C in 5% CO₂ for 24 or 96 hours. At 24 and 96 hours post-treatment, the medium was removed and cells were rinsed one time with PBS to remove dead and non-adherent cells. The cells were then treated with a fixing/staining solution containing 0.6% methylene blue (BBL, Cockeysville, MD) and 1.25% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in Hank’s Balanced Salt Solution (Invitrogen) at 37°C for 1 hour. Cells were rinsed with water and dried. The methylene blue stain was eluted from the cells with an elution solution containing ethanol/PBS/acetate acid 50:49:1 v/v/v by agitating the plate for 30 minutes. The optical density was measured by a Dynex Technologies MRX-II microplate reader (Dynex Technologies Inc, Chantilly, VA) at 570 nm. The optical density at 570 nm of all controls and treatments was fitted to a standard curve and used to express values as cell number.

2.7. Cytotoxicity

The cytotoxic effects of the controls and treatments towards Caco-2 cells were determined using the Non-Radioactive Cytotoxicity Assay Kit provided by Promega (Promega Corporation, Madison, WI). The assay assesses...
LDH activity in the culture medium as a result of cell lysis induced by the treatment. After treatment with phenolics extracts or controls for 24 or 96 hours, the culture medium was collected. Fifty microliters of substrate/enzyme mix were added to 50 µL of each sample. The solution was incubated at room temperature for 30 minutes protected from light. After 30 minutes, the reaction was stopped and the absorbance of each solution at 490 nm was recorded. Values were reported as percent LDH activity of Caco-2 cells grown under control growth conditions (see section 2.5. Cell culture).

2.8. Statistical analysis
Data were reported as mean ± standard deviation. ANOVA and Fisher’s comparison test were performed using Minitab Statistical Software v. 15 (State College, PA).

3. Results and Discussion
3.1. Total phenolic content of whole and refined wheat
The phenolic content from the insoluble-bound fraction of two commercial blends (Barretta and Magnolia) of whole and refined wheat was determined using the previously described Folin-Ciocalteu Reagent method. The total phenolic content of the insoluble-bound fraction of Magnolia and Barretta whole wheat was 95.8 and 97.5 mg/100 g, respectively (Table 1). The total phenolic content of the insoluble-bound phenolic fraction of Magnolia and Barretta refined wheat was 12.8 and 13.8 mg/100 g, respectively (Table 1).

The phenolic acid composition of whole and refined wheat was determined using an rp-HPLC-DAD method. Representative chromatograms from the insoluble-bound fraction of whole and refined wheat can be seen in Figure 2. The insoluble-bound ferulic acid content of Barretta and Magnolia whole wheat was 320 and 297 µmol/100 g, respectively. The insoluble-bound ferulic acid content of Barretta and Magnolia refined wheat was 30.8 and 19.3 µmol/100 g, respectively (Table 1).

Table 1: Phenolic acid composition of the insoluble-bound fraction of whole and refined wheat.
† Values expressed as mg GAE/100 g grain.
‡ Values expressed as µmol/100 g grain (mean ± standard deviation, n = 3).
Values with no letter in common within each column are significantly different (p < 0.05).
nd – not detected.

<table>
<thead>
<tr>
<th>Whole Wheat</th>
<th>TPC†</th>
<th>FA‡</th>
<th>p-CA‡</th>
<th>CA‡</th>
<th>VA‡</th>
<th>Σ phenolic acids‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barretta</td>
<td>97.5 ± 1.6 a</td>
<td>320 ± 19.4 a</td>
<td>18.2 ± 1.3 a</td>
<td>8.3 ± 1.0 a</td>
<td>nd</td>
<td>347 ± 20.8 a</td>
</tr>
<tr>
<td>Magnolia</td>
<td>95.8 ± 2.4 a</td>
<td>297 ± 15.5 a</td>
<td>17.0 ± 1.4 a</td>
<td>5.8 ± 1.1 b</td>
<td>nd</td>
<td>320 ± 17.8 a</td>
</tr>
<tr>
<td>Refined Wheat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barretta</td>
<td>13.8 ± 1.0 b</td>
<td>30.8 ± 11.2 b</td>
<td>7.5 ± 0.03 b</td>
<td>nd</td>
<td>nd</td>
<td>38.3 ± 11.1 b</td>
</tr>
<tr>
<td>Magnolia</td>
<td>12.8 ± 0.3 b</td>
<td>19.3 ± 5.9 b</td>
<td>7.0 ± 0.3 b</td>
<td>nd</td>
<td>nd</td>
<td>26.3 ± 6.2 b</td>
</tr>
</tbody>
</table>

Figure 2: Representative HPLC chromatograms from the insoluble-bound fraction of (a) whole wheat and (b) refined wheat. Chromatograms were monitored at 282 nm. See Methods section for further details on chromatographic separation of phenolic acids.
The insoluble-bound \( p \)-coumaric acid content of Barretta and Magnolia whole wheat was 18.2 and 17.0 µmol/100 g, respectively (Table 1). The insoluble-bound \( p \)-coumaric acid content of Barretta and Magnolia refined wheat was 7.5 and 7.0 µmol/100 g, respectively (Table 1).

Caffeic acid was only detected in the insoluble-bound fraction of whole wheat. The caffeic acid content of Barretta and Magnolia whole wheat was 8.3 and 5.8 µmol/100 g, respectively (Table 1). These values were significantly (\( p < 0.05 \)) different from one another.

### 3.2. Effect of phenolic acids and phenolic extracts from insoluble-bound fraction of whole and refined wheat on Caco-2 cell number

A broad range of cells were seeded and counted using the modified methylene blue stain assay as previously reported [16] and described in the Methods section of this paper and used to generate a standard curve for cell counting. The standard curve produced was similar to the previously reported standard curve [16] and had an \( R^2 \) value of 0.9919.

Caco-2 cells were seeded at densities of 4 x 10^4 and 2.5 x 10^4 cells/well in 96-well plates and growth curves were determined (Figure 3). There was a significant increase in the number of Caco-2 cells at 24 hours after cells were seeded when cells were seeded at a density of 4 x 10^4 cells/well. There was also a linear increase in the number of Caco-2 cells from 0 to 72 hours when cells were seeded at a density of 2.5 x 10^4 cells/well.

**Figure 3:** Caco-2 cell growth curve in 96-well plate. Caco-2 cells were seeded at densities of (a) 4 x 10^4 and (b) 2.5 x 10^4 cells/well in 5 separate 96-well plates, one plate for each time point.

Cells were counted using the modified methylene blue stain cell counting assay.

* indicates no significant difference (\( p > 0.05 \)) from the cell number 24 hours prior.

The total Caco-2 cell number after treatment with each phenolic acid treatment (2% DMSO v/v in growth medium) was assessed using a modification of the methylene blue stain assay at 24 and 96 hours post-treatment. The DMSO concentration used in this study was reported not to induce cytotoxic effects in Caco-2 cells [18]. Only \( p \)-coumaric acid significantly (\( p < 0.05 \)) reduced the number of Caco-2 cells at 96 hours post-treatment at a concentration of 50 µM compared to the number of cells when cells were grown under control growth conditions (Figure 4). Ferulic acid and caffeic acid significantly (\( p < 0.05 \)) reduced the number of Caco-2 cells at 96 hours post-treatment at a concentration of 500 µM compared to the number of cells when cells were grown under control growth conditions (Figure 4). The solvent control (2% DMSO in growth medium) did not reduce the number of Caco-2 cells at 96 hours post-treatment compared to the number of cells when cells were grown under control growth conditions.
Figure 4: Number of Caco-2 cells at 96 hours post-treatment with phenolic acids.

Caco-2 cells were seeded at a density of $2.5 \times 10^4$ cells/well. Six hours after seeding, the cells were incubated with phenolic acids (2% v/v DMSO in growth medium) at concentrations ranging from 25 to 500 µM. The cell number at 96 hours post-treatment was determined using the modified methylene blue stain assay (see Methods section for details). Values reported as mean ± standard deviation, n = 3. * indicates significant difference from the number of Caco-2 cells when cultured under control growth conditions (p < 0.05).

Caco-2 cell cultures were treated with phenolic extracts from the insoluble-bound fraction of whole and refined wheat at a concentration of 100 mg/mL. The total number of Caco-2 cells after treatment with phenolic extracts was assessed using the modification of the methylene blue stain assay at 24 and 96 hours post-treatment. Phenolic extracts from the insoluble-bound fraction of whole wheat significantly (p < 0.05) reduced the number of Caco-2 cells at 24 and 96 hours post-treatment compared to the number of Caco-2 cells grown under control growth conditions (Table 2).

The solvent control (10% v/v sterile water in growth medium) increased the number of Caco-2 cells at 24 or 96 hours post-treatment, though the increase in Caco-2 cell number was not significant (p > 0.05) (Table 2). The FBS control (growth medium without FBS) reduced the number of Caco-2 cells at 24 and 96 hours post-treatment but the reduction in cell number was not significant (p > 0.05).

The modified methylene blue stain assay has advantages over the more commonly used Trypan Blue stain cell counting method. First, significant (p < 0.05) differences in cell number can be observed 24 hours after plating the cells [16]. Use of the Trypan Blue stain method may indicate a false lag phase in cell growth at 24 hours. However, the modified methylene blue stain method shows consistent linear increase in cell number from 0 hours. Second, the methylene blue stain method has been modified for use in a 96-well plate [16], allowing for the use of less sample and fewer reagents.

The present study reports the abilities of the phenolic acids found in the insoluble-bound fraction of whole and refined wheat (ferulic acid, p-coumaric acid, and caffeic acid) to reduce Caco-2 cell number at 96 hours post-treatment. p-Coumaric acid had the most potent effect at 96 hours (Figure 4). Ferulic acid and caffeic acid also had significant (p < 0.05) effects on Caco-2 cell number at 96 hours post-treatment, however, the concentration needed to elicit this effect (500 µM) was higher than that of p-coumaric acid. This is consistent with the findings that ferulic acid did not inhibit the proliferation of HT-29 and SW480 colon cancer cells, in vitro [5] and did

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significantly inhibit the proliferation of Caco-2 cells at 72 hours post-treatment at a concentration 1500 µM [6]. The present study reports the significant (p < 0.05) antiproliferative activity of p-coumaric acid at 50 µM in Caco-2 cells. Phenolic acids have also been reported to reduce the number of non-epithelial cells but at lower concentration [19, 20].

Table 2: Effects of phenolic extracts from the insoluble-bound fraction of whole and refined wheat on Caco-2 cell number 24 and 96 hours post-treatment.

Caco-2 cells were seeded at a density of 4 x 10^4 cells/well for cell counting after 24 hours or 2.5 x 10^4 cells/well for cell counting after 96 hours. Six hours after seeding, the cells were incubated with growth medium, 10% v/v sterile water in growth medium, or 100 mg/mL insoluble-bound phenolics extract in 10% v/v sterile water in growth medium. The cell number was determined using the modified methylene blue stain assay described in the Methods section of this paper.

Values are reported as number of Caco-2 cells (mean ± standard deviation, n = 3). Percent medium control is in parentheses.
† Phenolic extracts were delivered at a sample concentration of 100 mg/mL.
‡ 10% v/v sterile water in growth medium.
* Within each column indicates a significant difference from the medium control at p < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>24 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell number (10^4)</td>
<td>Cell number (10^4)</td>
</tr>
<tr>
<td>Medium Control</td>
<td>7.3 ± 0.7</td>
<td>9.0 ± 0.8</td>
</tr>
<tr>
<td>Solvent Control ‡</td>
<td>7.6 ± 0.6 (104.2%)</td>
<td>9.5 ± 0.9 (104.2%)</td>
</tr>
<tr>
<td>Refined Wheat †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barretta</td>
<td>7.1 ± 1.3 (96.6%)</td>
<td>8.8 ± 1.1 (97.8%)</td>
</tr>
<tr>
<td>Magnolia</td>
<td>7.0 ± 1.6 (96.3%)</td>
<td>8.9 ± 0.9 (99.6%)</td>
</tr>
<tr>
<td>Whole Wheat †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barretta</td>
<td>4.6 ± 0.2 (59.9%) *</td>
<td>7.0 ± 2.1 (76.6%) *</td>
</tr>
<tr>
<td>Magnolia</td>
<td>4.5 ± 1.2 (59.0%) *</td>
<td>7.1 ± 1.4 (78.2%) *</td>
</tr>
</tbody>
</table>

The present study reports the ability of phenolic extracts from the insoluble-bound fraction of whole wheat, but not refined wheat, to reduce the number of Caco-2 cells at 24 and 96 hours post-treatment. The antiproliferative effect of phenolic extracts from whole grains or its milled fractions of whole grains is lacking. Hudson et al. reported that phenolic extracts from rice bran extract exhibited significant (p < 0.05) antiproliferative activity towards two colon cancer cell lines [5]. Another study reported the antiproliferative activity of the insoluble-bound fraction of whole and refined wheat in HepG2 cells [21]. In that study, phenolic extracts from the insoluble-bound fraction of both whole and refined wheat (10 mg/mL) significantly (p < 0.01) inhibited the proliferation of HepG2 cells. These data suggest that both whole and refined wheat are able to inhibit the proliferation of cell cultures, in vitro.

Janicke et al. (2005) noted that cells derived from an environment that is high in dietary components may be able to tolerate higher concentrations of dietary compounds [6]. This finding may partially explain why relatively low concentrations of phenolic acids and phenolic extracts were able to inhibit the proliferation of T47D breast cancer cells and ECV304 umbilical vein endothelial cells [19, 20]. Therefore, cells derived from the gastrointestinal tract may serve as better models for investigating the potential health benefit of whole grain phytochemicals in the lower gastrointestinal tract.

3.4. Cytotoxicity and cell death

The cytotoxic effect of phenolic extracts from the insoluble-bound fraction of whole and refined wheat was assessed as a marker of cytotoxicity using the Non-Radioactive Cytotoxicity Assay from the Promega Corporation. Cells were treated in the absence of phenol red and at a FBS concentration less than 5% v/v, as these two factors interfere with optical density readings by providing background absorbance. None of the treatments had significantly more LDH activity than the medium control at 24 hours post-treatment (Table 3).
To validate the assay, cells were grown in the absence of FBS and the cytotoxic effects of this treatment were assessed using the cytotoxicity assay. At 96 hours post-treatment, the FBS control (growth medium without FBS) had significantly (p < 0.05) more LDH activity than the medium control. Conversely, the solvent control (10% sterile water v/v in growth medium) had significantly (p < 0.05) less LDH activity than the medium control at 96 hours post-treatment (Table 3). At 24 and 96 hours post-treatment, the LDH activity of the whole wheat treatment was similar to that of the medium control. 

Table 3: Effects of phenolic extracts from the insoluble-bound fraction of whole and refined wheat on LDH activity in Caco-2 cells.

<table>
<thead>
<tr>
<th></th>
<th>24 Hours</th>
<th>96 Hours</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% medium control</td>
<td>% medium control</td>
<td></td>
</tr>
<tr>
<td>Medium Control</td>
<td>100 ± 21.9</td>
<td>100 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>Solvent Control</td>
<td>77.6 ± 5.8</td>
<td>57.9 ± 1.4 *</td>
<td></td>
</tr>
<tr>
<td>Refined Wheat</td>
<td>71.7 ± 3.2</td>
<td>80.8 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>Whole Wheat</td>
<td>94.9 ± 30.5</td>
<td>90.4 ± 5.1</td>
<td></td>
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</tbody>
</table>

In the present study, a cell counting assay and a cytotoxicity assay were used to assess the effect of phenolic extracts from the insoluble-bound fraction of whole and refined wheat on Caco-2 cell number. The cell counting assay showed that there was a significant increase in the number of Caco-2 cells at 24 and 96 hours after adding fresh culture medium to the cells (Figure 3).

Phenolic extracts from the insoluble-bound fraction of whole wheat significantly (p < 0.05) reduced the number of Caco-2 cells to 59.5% and 74.7% of the total number of cells when cells were grown under control growth conditions at 24 and 96 hours post-treatment, respectively (Table 3). However, there was no significant increase in cell death upon treatment with phenolics from the insoluble-bound fraction from whole wheat at 24 and 96 hours post-treatment (Table 4). These data suggest that the reduction in cell number upon treatment with phenolic extracts from the insoluble-bound fraction of whole wheat is due to the antiproliferative activity of the phenolic compounds from the insoluble-bound fraction of whole wheat and not due to increased cell death. This finding is supported by the reduction in cell number and increase in cell death when cells were cultured in the absence of FBS.

3.5. Limitations of the study
This study used an rp-HPLC-DAD assay that was only able to determine the most common phenolic acids found in whole and refined wheat using external standards. It is possible that other unidentified phenolic acids from the insoluble-bound fraction of whole and refined wheat may have significant antiproliferative activity. These phenolic acids include chlorogenic acid, o-hydroxybenzoic acid, methoxycinnamic acid, and sinapic acid. A more rigorous analytical method able to detect, identify, and quantify, these and other phenolic acids from the insoluble-bound fraction of whole and refined wheat may be needed to identify new compounds with significant antiproliferative activity.

4. Conclusion
The present study shows that phenolic extracts from the insoluble-bound fraction of whole wheat, but not refined wheat, can significantly (p < 0.05) reduce the number of Caco-2 colon cancer cells compared to cells grown under control growth conditions. This reduction in cell number is not due to the cytotoxic effects of the treatments. The reduction in cell number may be due to the antiproliferative effects of the treatments. This is the first study to...
report the antiproliferative activity of the insoluble-bound fraction of whole, but not refined wheat, in a human colon cancer cell line. Though the exact mechanism is unclear, the data suggest that phenolic acids found in the insoluble-bound fraction of whole wheat work synergistically to inhibit the proliferation of colon cancer cells. The data presented in this paper support epidemiological studies that have linked increased whole grain consumption to reduced risk of colon cancer [22, 23] and underscore the importance of whole grain consumption.

**Abbreviations**
ANOVA, analysis of variance; DMEM, Delbucco’s Modified Eagle’s Medium; DMSO, dimethyl sulfoxide; FA, ferulic acid; FBS, fetal bovine serum; GAE, gallic acid equivalents; HBSS, Hank’s Balance Salt Solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; p-CA, p-coumaric acid; p-HBA, p-hydroxybenzoic acid; PBS, phosphate buffered saline; rp-HPLC-DAD, reversed phase high-performance liquid chromatography-diode array detection; TPC, total phenolic content; VA, vanillic acid.

**Competing Interests**
The author has no competing interests. This research was conducted in partial fulfillment of the Doctor of Philosophy degree from Cornell University.

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**References**


