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Article

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Title: Effect of Fermented Wheat Germ Extract with *Lactobacillus plantarum dy-1* on HT-29 Cell Proliferation and Apoptosis

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ABSTRACT: This study aimed to evaluate the anticarcinogenic activities of aqueous extract of fermented wheat germ with *Lactobacillus plantarum* dy-1 (LFWGE). The anticarcinogenic activities, including antiproliferative effects and the induction of apoptosis, were studied in human HT-29 colon cancer cells. The 2, 6-dimethoxybenzoquinone and total phenols contents in LFWGE were determined by HPLC and the Folin-Ciocalteu method. In addition, some functional proteins were separated and purified by gel filtration chromatography. Twenty-one proteins were identified by LC-MS-MS. The sugars isolated from LFWGE did not possess any anticarcinogenic activity. The results of an MTT assay showed high antiproliferative effects of LFWGE. In addition, LFWGE attenuated the progression from the G_0–G_1 to the G_2–M phase of the cell cycle and LFWGE-induced cell apoptosis was associated with the activation of caspase-3. LFWGE and its major bioactive ingredients inhibited the proliferation of HT-29 cells via apoptosis and thus may be a potential anticarcinogenic agent.

KEYWORDS: Fermented wheat germ extract; 2, 6-dimethoxybenzoquinone; Protein; Apoptosis; Colon cancer

INTRODUCTION

The incidence of cancer is increasing worldwide.[1] Colon cancer, which is the presence of gastrointestinal malignant tumors, is conventionally treated by surgical resection, radiotherapy and chemotherapy. However, the cure rate is low and these treatments result in adverse patient reactions.[2,3] Current cancer treatments are now focused on agents that are more effective and less toxic. Evidence accumulated from several fields, including epidemiology, human medicine and nutrition, has indicated that dietary agents can safely regulate physiological function and enhance anticarcinogenic activity.[4,5] In addition, natural products have become more popular for the prevention and treatment of cancer.[6-8]

Wheat germ (embryonic axis and scutellum) represents about 2.5-3.8% of the total seed weight and is an important by-product of the flour milling industry.[9] It is systematically removed during milling because it adversely affects the shelf-life and quality of the flour. However, due to the abundance of protein, fat, vitamins and other nutrients in wheat germ, it is praised by nutritionists as a "natural nutrient treasure-house and life source of mankind."[10] Numerous products can be developed from wheat germ, including wheat germ oil;[11] protein foods such as processed meat;[12] extruded high-protein foods and beverages;[13] and and other functional foods such as *nutritive noodles*,[14] cereals and baked goods.[15,16] However, currently, about 2,000,000-2,500,000 tons of wheat germ is not fully exploited in China and other parts of the world. There are two main reasons for this phenomenon. First, wheat germ has a poor shelf life due to the presence of unsaturated fatty acids and high lipase and lipoxygenase activity that rendering the product highly susceptible to rancidity.[17] Second, it has also some anti-nutritional factors such as phytic acid, raffinose and wheat germ agglutinin. [18]

Recently, cereal fermentations have shown significant potential in the improvement and design of the nutritional quality and health effects of foods and ingredients.[19,20] The hot-spots for wheat germ research are shifting from principal component extraction to its microbiological transformation. The fermentation of wheat germ has been used to enhance its nutritional and functional properties. During the fermentation of wheat germ with probiotic lactobacilli and bifidobacteria, prebiotic compounds have been liberated.[21] Rizzello et al.[18] exploited the potential of sourdough lactic acid bacteria (*Lactobacillus plantarum* LB1 and *Lactobacillus rossiae* LB5) to improve the stabilization of wheat germ and reduce its anti-nutritional factors such as phytic acid and raffinose. In addition, Rizzello et al.[22] also studied the use of sourdough-fermented wheat germ (SFWG) for enhancing the nutritional, textural and sensory characteristics of white bread. Rizzello et al.[23] showed that SFWG possesses marked antifungal activity that may extend the microbial shelf-life of leavened baked goods.

Several studies have shown that Saccharomyces cerevisiae (yeast)-fermented wheat germ extract (MSC, Trade name: Avemar) also possesses antioxidant and anti-inflammatory properties,[24] and displays anti-carcinogenic activities in various cancer models such as testicular, colon, NSCLC, melanoma, leukemia and gastric cancer.[25,26] However, the exact chemical composition of Avemar is not known.[27] The yeast fermented wheat germ extract (YFWGE) contains hundreds to thousands of different molecules, but based on recent studies with various extracts from fermented wheat germ, it is currently assumed that the two quinones present in wheat germ as glucosides—2-methoxy benzoquinone(2,6-DMBQ) and 2, 6-dimethoxy benzoquinone(2-MBQ)—are probably responsible for some of the biological properties of YFWGE.[27] However, the data from Hidvegi et al. obtained using a skin

graft model indicates that components other than the two benzoquinones are responsible for the immune stimulatory activity of YFWGE.[26,28] Furthermore, Tuscano et al.[29] showed that YFWGE contains proteins (polypeptides) with molecular weights of 5-100 Kilo Daltons (KD) that are active in inhibiting the proliferation of cancer cells. Thus, there is interest in developing wheat germ using biotechnology with lactic acid bacteria and yeast.

Although wheat germ fermentation has been widely studied, there is little information about the anticarcinogenic activity of wheat germ extract fermented with lactic acid bacteria. In 2013, lactic acid bacteria isolated from wheat germ were characterized and selected based on technological performance to be used as starters for fermenting wheat germ, and an Italian researcher found that the fermented wheat germ extract could inhibit the growth of the colorectal cancer cell lines HT-29, HCT-8 and DLD-1.[30] However, only the benzoquinones of the fermentation extracts were analyzed, and thus further research is needed on other anticarcinogenic components.

In this study, we evaluate the anticarcinogenic activities of *Lactobacillus plantarum* dy-1 fermented wheat germ extracts (LFWGE), including the growth inhibition and induction of apoptosis via the Caspase-3 signaling pathway in the human colon cancer HT-29 cell line. In addition, some of the compounds in LFWGE were analyzed and identified.

MATERIALS AND METHODS

Materials and Chemicals. The fresh wheat germ was purchased from Shandong Yongle of China. The moisture, ash and fat content were determined according to the Approved Methods of the American Association of Cereal Chemists.[31] The crude protein content (Kjeldahl method) was measured with a semi-automatic Kjeldahl apparatus (K355, Buchi, Switzerland) and a Metrohm 877 Titrino Plus Automatic

Titrator (Switzerland). A conversion factor of 5.70 was used. The lactobacillus strain *Lactobacillus plantarum* dy-1 was previously isolated by authors. HT-29 colon cancer cells were provided by the Cell Bank at the Chinese Academy of Sciences in Shanghai. Foetal bovine serum and McCOY'S 5A medium were purchased from GIBCO. Trypsin-EDTA was purchased from Beyotime. Annexin V-FITC, propidium iodide (PI), Hoechst dye 33342, penicillin, chloramphenicol, 2, 6-dimethoxybenzoquinone, gallic acid, Folin-Ciocalteu reagent, coomassie brilliant blue R250 and bovine serum albumin were purchased from Sigma-Aldrich, Inc. (Supelco, Bellefonte, PA, USA). The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), sephadex G-50 and sephacryl S-200 were purchased from Shanghai Chuangsai Scientific Instrument Limited Co. (Shanghai, China). All of the other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and were of either analytical or chromatographic grade.

The Preparation of the Extracts. *Lactobacillus plantarum* dy-1 was used as the starter culture in the direct vat set fermentation, which was previously isolated from pickles by authors. Its preservation number is CGMCC NO. 6016 in the Chinese Common Microbe Bacterial Preservation Administration Center database. *Lactobacillus plantarum* dy-1 was cultivated in MRS, treated with a protective agent (20% skim milk, 10% fucose, 14% monosodium glutamate, 6% sorbitol and 6% Vitamin C) and Lyophilized to powder. The wheat germ was crushed and passed through a 100-mesh sieve. Two hundred grams of wheat germ powders, 1.4 L of distilled water and 4 g of freeze-dried fungus powder containing *Lactobacillus plantarum* dy-1 (cell density 4×10^8 cfu/g) were shaken and incubated at 30 °C for 24 h in a microbiological incubator. After fermentation, the solution was centrifuged at 12,000 g for 15 min at 4°C using a refrigerated centrifuge (Jouan, France), then the

supernatant was freeze-dried into a powder (LFWGE) using a vacuum freeze dryer (Marin Christ, Germany). For the unfermented wheat germ, 200 g of powder were extracted with 1.4 L of distilled water at room temperature for 3 h in shaking tables. The supernatant was collected by centrifuging, and then freeze-dried into a powder (WGE) under the same conditions as the fermented wheat germ. This step was repeated six times for analysis. The freeze-dried samples were stored in sealed containers at -20°C for further analysis. The extraction yield was calculated by the following equation: Extraction yield (%) = [weight of freeze dried powder / weight of WG (g)] ×100.

Extraction of Benzoquinones and HPLC Analysis. The benzoquinones were extracted and analyzed using the following procedure. About 0.5 g of WGE or LFWGE was dissolved into 50 mL of double-distilled water and subjected three times to extraction by shaking with 25 mL of chloroform ($CHCl_3$).[32] The $CHCl_3$ layers were pooled, washed three times with distilled water and dried over anhydrous Na_2SO_4. The solvent was evaporated to dryness by rotary evaporation at 40°C. The residue was redissolved in 5 mL $CHCl_3$ and filtered through a 0.22 μm filter membrane. The filtrate was analyzed by a High Performance Liquid Chromatography (HPLC) system consisting of an Äkta purifier HPLC equipped with shim-pack VP-ODS (250 mm × 4.6 mm, 5 μm) and an ultraviolet detector operating at 288 nm. The mobile phase was a water:methyl alcohol mixture (80:20, v/v), with the flow rate and sample injection volume fixed at 1mL/min and 20 μL, respectively. The temperature was 25°C. 2, 6-dimethoxybenzoquinone (Sigma) dissolved in 100% $CHCl_3$ was used as reference to assign retention times and generate the calibration curve

Determination of the Protein Content and SDS-PAGE Analysis of the Extracts. The protein content of the extracts was determined using the Kjeldahl

153 method. A conversion factor of 5.70 was used.

154 The size and purity of the extracted protein were analyzed by sodium dodecyl
155 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed as
156 follows. The aqueous solutions of LFWGE and WGE were mixed with a quarter
157 volume of sample buffer (10% SDS, 0.5% bromophenol blue, 0.25M Tris-HCl, pH6.8,
158 50% glycerin and 5% β-mercaptoethanol) and heated in a water bath at 100°C for 5
159 min. After centrifugation (6000 × g for 10 min), 10 μL of the supernatant was
160 electrophoresed with 12% gel concentration and mini-cell apparatus (Bio-Rad) at
161 20mA for 3 h and stained with Coomassie brilliant blue R-250 for 2 h followed by
162 destaining using methanol/acetic acid/water at a ratio of 10:7.5:82.5. A GS-800
163 calibrated densitometer (Bio-Rad) was used to scan the gel.

164 **Separation and Identification of Proteins in the LFWGE.** The components of
165 the LFWGE, which were concentrated by precipitation with solid ammonium sulfate
166 to 80-90% saturation, were separated and purified. After being centrifuged (12,000 g
167 for 15 min at 4°C), the precipitate was redissolved in distilled water and dialyzed
168 (molecular weight cut off: 8000-10000 Da) against distilled water at 4°C for 48 h
169 (renewal of distilled water every other 4-8 h), and finally freeze-dried. Then, the
170 freeze-dried powder was dissolved in phosphate buffer (pH 7.2, 10 mmol/L) and the
171 solution was passed through a sephadex G-50 column (1.6 cm × 50 cm) in series with
172 a sephacryl S-200 (1.6 cm × 50 cm) column at 1 mL/min equilibrated and eluted with
173 phosphate buffer (pH 7.2, 10 mmol/L). The eluent was monitored at 280 nm by UV
174 absorbance, and fractioned peaks were collected, dialyzed against distilled water and
175 freeze-dried for the assay of anticarcinogenic activity. The anticarcinogenic fractions
176 of the eluent were analyzed by liquid chromatography and tandem mass spectrometry
177 (LC-MS/MS) analysis at the Institute of Biochemistry and Cell Biology, Shanghai

Institutes for Biological Sciences (SIBS) of the Chinese Academy of Sciences (CAS).

RP-HPLC was performed using a surveyor LC system (Thermo Finnigan, San Jose, CA) on a C18 column (RP, 180 μm × 150 mm, BioBasic® C18, 5μm, Thermo Hypersil-Keystone). The pump flow rate was 2 μL/min. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The protein mixtures were eluted using a gradient of 5-65% B over 30 min. The mass spectral data were acquired on an LTQ linear ion trap mass spectrometer (Thermo Finnigan) equipped with an electrospray interface operated in positive ion mode. The temperature of the heated capillary was set at 200℃. A voltage of 3.2 kV applied to the ESI needle resulted in a distinct signal. The mass spectrometer was set so that one full MS scan was followed by three MS/MS scans on the three most intense ions from the MS spectrum with the following Dynamic Exclusion TM settings: repeat count, 2; repeat duration, 0.5 min; exclusion duration, 2.0 min.

Determination of the Total Phenols Content. The total phenol content in WGE and LFWGE was determined using the Folin-Ciocalteu method, slightly modified according to Heimler et al.[33] About 0.5 g of WGE or LFWGE was weighed in a 100 mL volumetric flask and dissolved in 40% ethanol aqueous solution. Then, 1 mL of the solution was added to 4 mL of deionized water and 1 mL of Folin-Ciocalteu reagent in glass test tubes. After the mixture was shaken, 10 mL of a 7% aqueous Na_2CO_3 solution was added and the mixture was shaken once again. The final volume was adjusted to 20 mL with distilled water. After 90 min of reaction at 45℃, the absorbance at 765 nm using a spectrophotometer (model UV-9600, Rayleigh, Beijing, China) with water as a blank was measured and used to calculate the phenol content, using gallic acid as a standard. The total phenol amount was expressed as gallic acid equivalent (GAE, mg gallic acid/g sample) through the calibration curve of gallic acid.

The calibration curve ranged from 20 to 100 μg/mL (R^2= 0.9924).

Determination and Extraction of the Total Sugar Content. The total polysaccharide content was determined by the phenol-sulfuric acid method with slight modifications.[34] First, 2 mL of the sample solution was vortex-mixed with 1 mL of 5% phenol in water before rapidly adding 5 mL of concentrated sulfuric acid. After 30 min of standing at room temperature, the absorbance of the sample solution was measured at 490 nm against the blank, which was prepared by substituting distilled water for the sample solution. Aqueous glucose solutions of different concentrations (5, 10, 20, 40, 80 and 160 μg/mL) were used for the standards. The results were expressed as grams of glucose per 100 g of extract.

Crude polysaccharides were extracted with hot water from the LFWGE. The extraction procedure was as follows. First, 25 g of LFWGE was dissolved in 150 mL of double-distilled water, heated at 100°C for 10 min and then filtered. The clear solution was concentrated in a rotary evaporator under reduced pressure at 50°C. Then, the concentrated solution was precipitated by the addition of absolute ethanol (4 times the volume of concentrated solution) at 4°C, followed by centrifugation at 4,800 × g (20 min) to yield the crude polysaccharides. Then, the deproteinization of the crude polysaccharides was performed using the Sevag method.[35] Finally, the polysaccharide samples were freeze-dried into a powder.

Determination of Lactic Acid Content. The lactic acid content in the LFWGE was determined by high performance liquid chromatography (HPLC). The samples were dissolved in distilled water (about 1.0 mg/mL) and filtered through a 0.45 μm PTFE membrane filter. The WGE or LFWGE was analyzed by a Shimadzu LC-20A HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a photo-diode array (PDA). The operating conditions included a shim-pack VP-ODS C-18 (250 × 4.6 mm,

5 μm) column (Shimadzu Corp., Kyoto, Japan), a mobile phase of 3% methanol/0.01 mol/L K_2HPO_4 (pH 2.5), a volume injection of 20 μL, a flow rate of 0.8 mL/min and detection at 210 nm at 25°C. The standard curves for lactic acid (Sangon Biotech Co., Ltd., Shanghai, China) were used to quantify the acid.

Cell Lines and Cell Culture. The human colon cancer cell line HT-29 was used for experimentation and obtained from the Shanghai Institutes for Biological Sciences (SIBS) of the Chinese Academy of Sciences (CAS). The HT-29 cells were cultured as monolayers with up to 80% confluence in McCOY'S 5A supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin at 37°C and 5% CO_2 in humidified air. Cell counts were determined using a micro-cell counter CC-108 (Sysmex, Kobe, Japan) and cells in a logarithmic phase of growth were used for all of the studies described below.

Growth Inhibition Assay. Cell growth was determined by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.[5] The HT-29 cells were placed in 96-well plates 24 h before treatment. Following treatment with 125-4000 μg/mL of LFWGE (adjustment of pH to 7.0 with 0.1M NaOH) and WGE (control), the proliferation activity of the cells was tested after 24, 48 and 72 h of incubation. Following treatment with 12.5-100 μg/mL of 2,6-dimethoxy benzoquinone (standard), 25-200 μg/mL of protein fraction, 250-4000 μg/mL of sugar and 25-100 μg/mL of lactic acid (standard), respectively, the proliferation activity of the cells was measured after 24 h of incubation.

Absorbance was measured at 490 nm using a Multiskan Spectrum (Thermo Electron Corp., Asheville, NC). Cell viability was expressed as a percentage of the control culture value, which was considered to be 100% viable.

Cell Cycle Distribution Analysis. The HT-29 cells (0.4×10^6 per mL) were

seeded in 6-well culture plates and incubated with increasing concentrations of LFWGE (0 (double-distilled water), 0.2, 0.4, 0.8 mg/mL) at 37°C under cell culture conditions. After 48 h, the cells were harvested and suspended in 5 mL of cold PBS, centrifuged (500 g for 10 min) and resuspended and fixed in 3 mL of cold ethanol (70%) for overnight at 4°C. After washing twice with cold PBS, RNAse A and propidium iodide were added to a final concentration of 50 μg/mL each. The sample was then incubated at 4°C for 30-60 min before measurement. The cells were analyzed on a flow cytometer (Beckman Coulter, USA), and the cell cycle distribution was calculated with the ModFit LT software (Verity Software House, Topsham, ME, USA).

Hoechst Dye 33342 Staining. The HT-29 cells (0.4×10^6 per mL) were seeded in 12-well culture plates and exposed to 1 mg/mL concentrations of LFWGE for 48 h. Hoechst 33342 was added directly to the cells to a final concentration of 5 μg/mL. After 15 min at room temperature in the dark, the cells were visualized with a Leica DMR XA fluorescence microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) equipped with the appropriate filters for Hoechst 33342 to determine the nuclear morphological changes.

Assessment of Apoptosis by Flow Cytometry. Apoptosis was determined with an apoptosis kit (Sigma). The procedure was performed according to the manufacturer's instructions. Briefly, after treatment with different concentrations(0.25, 0.5, 1, 2 and 4 mg/L) of LFWGE for 24, 48 and 72 h, the HT-29 cells were harvested and collected by centrifugation. The cells were washed with ice-cold PBS and resuspended in the binding buffer at a concentration of 10^6 cells/ml. Then, 500 μL of cell suspension was mixed with 5 μL of annexin V-FITC and 10 μL of Propidium Iodide (PI) solution. The mixtures were incubated for 10 min at 4°C and protected

from exposure to light. The fluorescence of the cells was analyzed by flow cytometry. Approximately 2×10^4 cells were tested for each histogram by flow cytometry.

Quantitative PCR. Approximately 2×10^7 HT-29 cells were lysed in Trizol Reagent. Total RNA was prepared according to the manufacturer's instructions (Takara, Japan). To remove the genomic DNA, 5 μg of total RNA was treated with 5U RNAse-free DNAseI for 30 minutes at 37°C. After the DNAse treatment, the RNA were incubated at 65°C for 10 minutes. RNA integrity was assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Quantitative PCR was used to generate RNA with two sharp ribosomal 18S and 28S bands. First-strand cDNA synthesis was carried out on 2 μg of the total RNA from each sample with the PrimeScript® RT Master Mix (Takara, Japan) first-strand synthesis kit for RT-PCR according to the manufacturer's instructions. Experimental wells containing 25 μL of SYBR Green PCR Master Mix (Takara, Japan) were run using north tube plates. Quantitative PCR was conducted on the iCycler according to the SYBR Green method. Forward and reverse primers were designed using the tools available through the MIT Whitehead Institute web page. The primer sequences were as follows: caspase-3(F) AACCTCAGGGAAACATTCAG, caspase3(R)GGCTCAGAAGCACACAAAC;caspase8(F)GGATGCCTTGATGTTAT TCC,caspase8(R)AGTTCCCTTTCCATCTCCTC;caspase9(F)TTCCCAGGTTTTGT TTCCTG,caspase9(R)ACCCTAAGCAGGAGGGACTG;caspase7(F)GAAGAGGCT CCTGGTTTGTG,caspase7(R)TCATGGAAGTGTGGGTCATC;Actin(F)AGCGAGC ATCCCCCAAAGTT; and Actin(R) GGGCACGAAGGCTCATCATT. Quantitative PCR amplification for all of the caspases included preincubation at 94°C for 6 min, followed by 38 cycles at 95°C for 5 s, 57°C for 45 s and 72°C for 30 s. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. [36]

Caspase-3 Activity. The caspase-3 activity was determined using a Caspase-3 activity kit (Nanjing Jiancheng Bio-engineering Research Institute, Nanjing, China). Cell lysates were prepared after their respective treatment with various designated procedures and the assays were performed on 96-well microtitre plates by incubating 10 μL of cell lysate protein per sample in 80μL reaction buffer (1% NP-40, 20 mM Tris-HCl (PH 7.5), 137 mM Nad and 10% glycerol) containing 10μL of caspase-3 substrate (Ac-DEVD-pNA) (acetyl-Asp-Glu-Val-Asp p-nitroanilide) (2 mM). The lysates were incubated at 37°C for 4 h. The samples were measured with an ELISA reader at an absorbance of 405nm by a spectrophotometer.

Statistical Analysis. All of the statistical analyses were accomplished by using SPSS Statistics software version 17.0 (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was used to test for differences between the means.

RESULTS AND DISCUSSION

Yield and Chemical Composition of the Extracts. The composition of raw wheat germ was as follows: moisture 12.48 ± 0.46%, crude protein (N×5.70) 37.88 ± 0.24% of dry matter (d.m.); fat 11.91 ± 0.34% of d.m.; and ash 7.68 ± 0.09% of d.m. In this experiment, the yields of the aqueous extract of LFWGE and WGE were 22.19 ± 1.32% and 40.02 ± 1.82%, respectively.

Table 1 presents the composition of LFWGE and WGE. As shown, WGE contained 32.90 ± 0.57% of crude protein and 43.05 ± 1.23% of total sugar. After 24 h of incubation with *Lactobacillus plantarum* dy-1 (initial cell density 4×10^8 cfu/g) at 30°C (the optimum fermentation condition according to some preliminary work for antitumor activity), the protein in LFWGE slightly increased to 34.36 ±0.23% and the sugar decreased to 33.04±1.03%. In addition, the total phenol content of WGE was 20.81±0.11 mg/g (expressed as the gallic acid equivalent, mg gallic acid/g dried

328 extract). The total phenol content of LFWGE was to 28.48 ± 0.16 mg/g. Lactic acid
329 was not detectable in WGE, whereas 27.7 ± 1.2 mg/g of lactic acid was found in
330 LFWGE.

331 Due to the difficulty of buying 2-methoxy benzoquinone in China, 2,
332 6-dimethoxybenzoquinone was selected as the analysis target. The 2,
333 6-dimethoxybenzoquinone content in WGE and LFWGE was then determined by
334 $CHCl_3$ selective extraction and HPLC analysis. WGE contained 2,
335 6-dimethoxybenzoquinone at a concentration of 33.82 ± 2.85 μg/g. After fermentation,
336 the concentration of 2, 6-dimethoxybenzoquinone in LFWGE increased to 181.10 ±
337 4.56 μg/g (Table 1). This result shows that the fermentation had a significant effect on
338 the 2, 6-dimethoxybenzoquinone content of the aqueous extract of wheat germ, which
339 may be attributable to the release of benzoquinones present in wheat germ as
340 glucosides, as found by Rizzello et al.[30] and Yoo et al.[37].

341 **Inhibition of Cell Proliferation in LFWGE-treated HT-29 Cells.** To examine
342 the effects of LFWGE and WGE on cell growth, their inhibitory capacity against
343 HT-29 colon cancer cells was assessed using the MTT assay. Logarithmically growing
344 HT-29 cells were seeded at a concentration of 3×10^4 cells per mL and then HT-29
345 colon cancer cells were treated with different concentrations of LFWGE and
346 WGE(0.125-4 mg/mL) for 24, 48 and 72 h. As shown in Figure 1, WGE hardly
347 inhibited cell proliferation in the HT-29 cells. However, with increasing
348 concentrations of LFWGE, an increasing inhibition of cell proliferation in HT-29 cells
349 was observed, with different degrees of potency between incubation times. The IC_{50}
350 value was defined as the concentration at which the cell proliferation was inhibited by
351 50% of the control cells. LFWGE inhibited the growth of HT-29 cells in a dose- and
352 time-dependent manner with IC_{50} values of 1.288, 0.6397 and 0.5435 mg/mL,

353 respectively. These results indicate that LFWGE displays anti-carcinogenic activity in
354 HT-29 colon cancer cells.

355 Several studies have described the promising anticarcinogenic activity of a yeast
356 (Saccharomyces cerevisiae) fermented wheat germ extract (Avemar®), which is
357 attributed to two quinones: 2,6-dimethoxybenzoquinone (2,6-DMBQ) and 2-methoxy
358 benzoquinone (2-MBQ).[27,28] Yoo et al.[37] and Rizzello et al.[31] also demonstrated that
359 both benzoquinones could be obtained from wheat germ using lactic acid bacteria
360 fermentation. The HT-29 cells were treated with multiple levels of 2,6-DMBQ
361 isolated from LFWGE to determine the dose-dependent response to this compound.
362 The results obtained, shown in Table 2, show that the cells proliferation inhibition rate
363 increased with the concentration. However, combined with the results of LFWGE
364 treatment and 2,6-DMBQ content, we found that the proliferation of HT-29 cell was
365 inhibited not only by benzoquinones. Therefore, further research on other functional
366 components is still needed.

367 Some studies have also exploited the potential of specific low molecular proteins
368 or polypeptides to reduce or inhibit the growth or proliferation of a cancer cell.[29]
369 Proteins played a role in this study, and their effects on the cancer cells by isolated
370 protein fractions from LFWGE were analyzed. Electrophoresis (SDS-PAGE) of WGE
371 and LFWGE was performed to obtain information on the molecular weight and
372 distribution patterns of the protein components (Figures 2a and b). Protein bands were
373 observed distinctly between 15 and 120 kDa in WGE, and between 15 and 40 kDa in
374 LFWGE.

375 The extracted proteins were isolated and purified by sephadex G-50 and
376 sephacryl S-200 gel filtration. To improve the separation efficiency, series columns
377 were adopted. A typical elution profile is shown in Figure 3. The elution profile was

reproducible and revealed two main peaks (due to collection and detection difficulties, other smaller peaks were ignored). Among them, the peak1 fraction indicated the highest anticarcinogenic activity inhibiting the growth of HT-29 cells with 88.6% in 200 μg/mL (Table 2). An LC-MS/MS analysis was carried out for peak 1, and 21 proteins were observed and identified by searching against the NCBInr viridiplantae protein database. The active proteins were added to those listed in Table 3.

In addition, to determine which sugars in LFWGE might be at work, sugars were extracted and treated on HT-29 cells to trace any activity. The result showed no anticarcinogenic activity (Table 2).

It is important to note that the pH was adjusted to 7.0 with 0.1 M of NaOH in vitro treated with LFWGE to eliminate the influence of low pH, which was attributed to the production of lactic acid during fermentation. The effect of lactic acid in the HT-29 cell lines was studied, but the result showed that it had no significant effect on the HT-29 cells (Table 2).

Cell Cycle Distribution after Treatment with LFWGE. To further confirm the proliferation inhibiting effect of LFWGE on HT-29 cells, the cell-cycle distribution was analyzed by flow cytometry. Preliminary experiments have shown that while the cells die within 72 h, they do not change significantly for 24 h, so 48 h treatment was selected. The effect of LFWGE on the cell-cycle distribution of HT-29 cells is shown in Figure 4. In the control cells, the cell cycle pattern remained constant over time. After 48 h of incubation with increasing doses of LFWGE, the percentage of cells in each phase was as follows: G_0/G_1 phase (DNA presynthetic phase)—61.2, 70.2 and 80.8%; S phase (DNA synthesis phase)—28.4, 25 and 14.8%; G_2/M phase (DNA post-synthetic and mitosis phases)—10.5, 4.78 and 4.34%, respectively. Overall, a broad peak appeared in the G_0/G_1 region with a significant decrease in the S cycle

phase. Therefore, LFWGE halted the cell cycle transition of HT-29 cells in the G_0/G_1 phase of the cell cycle, resulting in the depletion of G_2/M phase cells. Studies have shown that Avemar also stops the cell-cycle transition of HT-29 cells in the G_0/G_1 phase of the cell cycle and halts the cell cycle transition of HL-60 cells in the G_2/M phase of the cell cycle.[38]

Induction of Apoptosis by LFWGE. To evaluate the effect of LFWGE on the induction of apoptosis, the HT-29 cells were incubated with 0.25, 0.5, 1, 2 and 4 mg/mL of LFWGE for 24, 48 and 72 h, and then double stained with annexin V-FITC and PI, followed by quantitative flow cytometry analysis. The results confirmed that LFWGE induced the apoptosis of HT-29 cells, which was positively correlated with LFWGE dose and incubation time (Figure 5). The cells were double stained with annexin V-FITC and propidium iodide and analyzed by flow cytometry to examine the distribution of late and early apoptotic cells. The control cells demonstrated normal cell viability without significant cell apoptosis. In contrast, after treatment with 1 mg/mL of LFWGE, the portion of normal cells was only 2.6%, whereas the proportion of early apoptotic cells was 68.4% and that of late apoptotic cells was 28.9%. From these results, we found that LFWGE functions similarly to Avemar. It has been previously shown that Avemar treatment leads to an increase in apoptosis.[31,39] However, with regard to potency, the former is more effective than the latter.

Cell death in multicellular organisms exhibits two patterns: necrosis and apoptosis. Apoptosis is a process of programmed cell death characterized by various biochemical and morphological changes, including cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and the formation of “apoptotic bodies.”[40] To further elucidate apoptotic cell death due to the exposure of HT-29 cells

to LFWGE, the cells were stained with Hoechst dye 33342 and examined using a Leica DMR XA fluorescence microscope. As shown in Figure 6, the control cells exhibited intact and round-shaped nuclei characterized by a diffused chromatin structure (a). In contrast, exposure to 1 mg/mL of LFWGE for 72 h significantly altered the HT-29 cell morphology; that is, the nucleus exhibited condensed chromatin (b), which is characteristic of apoptotic cells. Moreover, combined with the cell cycle distribution, we found that the cell nuclei of early apoptotic cells were pycnoid or round-bead shaped, whereas those of late apoptotic cells appeared to have apoptotic bodies (Figure 7).

Role of Caspases in the Apoptotic Effect of LFWGE. Apoptosis is a well-controlled process involving a programmed set of cellular events partially mediated by caspases, which are the cysteine aspartate proteases involved in apoptosis that lead to the loss of cellular structure and function, phosphatidylserine exposure and ultimately cell death.[41] Initiator caspases, such as caspases-2, -8 and -9, are closely linked to pro-apoptotic signals.[42] The effector caspases-3, -6 and -7 cleave cytoskeletal and nuclear proteins to induce apoptosis.[43,44] We therefore approached the question of caspases involvement in HT-29 cell processes by assaying the transcriptional regulation of caspases mRNA. The RNA purity was quantified by spectrophotometer with a OD260/280 ratio of 1.8 to 2.0 for all specimens. The RNA integrity was determined by 1% agarose gel electrophoresis, yielding two sharp ribosomal 18S and 28S bands. The expression of caspase-3, caspase-7, caspase-8 and caspase-9 was presented as $2^{-\Delta\Delta Ct}$. caspase-3, caspase-7, caspase-8 and caspase-9 mRNA expression at 48 h after the addition of 0.4 mg/mL and 0.8 mg/mL of LFWGE was detected by qPCR. Following the treatment of the HT-29 cells with increasing doses of LFWGE, the mRNA expression levels of caspase-3 ($F = 163.59$, $P = 0.000$),

caspase-7 (F = 504.491, P = 0.000), caspase-8 (F = 403.52, P = 0.000) and caspase-9 (F = 1465.930, P = 0.000) significantly increased compared with the control group (Figure 8).

Caspase-3 is an executioner caspase activated by multiple pathways[45] that has been shown to increase with Avemar treatment.[32] The treatment of HT-29 cells with 0.4, 0.8 and 1.5 mg/mL of LFWGE for 48 h induced a dose-dependent activation of caspase-3. LFWGE induced a detectable increase in caspase-3 activity after 48 h of treatment, showing a 1.6, 2.6, and 4.08 -fold increase, respectively (Figure 9). Therefore, growth inhibition and the induction of apoptosis in tumor cells may be associated with the increased expression of caspases.

In conclusion, our findings confirm the value of LFWGE as a natural product with anticarcinogenic properties. LFWGE as a strong regulator is able to inhibit the growth of HT-29 cells and induce apoptosis in vitro. Further research is required to identify which components or which group of low molecular weight proteins are the most pronounced functional ingredients. In addition, the results indicate the possible involvement of a coordinated caspases response to induce apoptosis. Based on our data, LFWGE might warrant further investigation in the prevention or treatment of human colon cancer.

ABBREVIATIONS USED

KD, Kilo Daltons; LFWGE, Aqueous extract of fermented wheat germ with *Lactobacillus plantarum* dy-1; WGE, Aqueous extract of raw wheat germ; SFWG, Sourdough fermented wheat germ; YFWGE, Yeast fermented wheat germ extract; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PI, Propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide; ANOVA, Analysis of variance.

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Figure Captions

Figure 1. HT-29 cell proliferation in response to LFWGE and WGE treatment. Colon cell cultures were treated with increasing doses of LFWGE as indicated on the x axis. Cell proliferation was determined by formazan dye uptake and expressed as a percent of untreated control cell proliferation. Mean ±SD, n = 8.

Figure 2. SDS-PAGE patterns of proteins in WGE and LFWGE: WGE1 (0.1 mg), WGE2 (0.2 mg), LFWGE1 (0.1 mg), LFWGE2 (0.2 mg).

Figure 3. Chromatography of LFWGE on Sephadex G-50 column and sephacryl S-200 column, eluent: phosphate buffer (pH 7.2, 10 mmol/L); flow rate, 1mL/min.

Figure 4. HT-29 cell cycle changes in response to LFWGE treatment (48 h). HT-29 cell cultures were treated with increasing doses of LFWGE as indicated on the right column, and cell cycle distribution was determined using flow cytometry after PI staining, and expressed as a percent of G_0/G_1, S, and G_2/M cycle phases. The DNA histograms show that LFWGE induced a dose-dependent decrease in the S cycle phase, whereas there was a significant expansion of the G_0/G_1 cycle phase consistent with an increase in the number of apoptotic HT-29 cell figures. Analysis by flow cytometry showed the distinct signals and cell frequencies associated with the arrested cell cycle status as described under "Results" (n = 6).

Figure 5. HT-29 cell apoptosis in response to LFWGE treatment. HT-29 cell cultures were treated with increasing doses of LFWGE as indicated on the x axis, and the number of apoptotic HT-29 cell was determined using flow cytometry after PI and annexin V staining. Mean ±S.D. n = 3, *, $p < 0.05$, **, $p < 0.01$.

Figure 6. Effect of LFWGE treatment on the nuclear morphological changes of HT-29 cells. (a) control cells; (b) cells treated with 1 mg/mL LFWGE for 72 h. The medium was removed, and the cells were incubated with 5 μg/mL Hoechst for 15 min

642 at room temperature in the dark. Stained solution was washed out, and cells were
643 visualized under fluorescence microscopy (200×).

644 **Figure 7.** HT-29 cell early apoptosis and late apoptosis in response to LFWGE
645 treatment using flow cytometry. HT-29 cell cultures were treated with 1 mg/mL
646 LFWGE (72 h), and the formation of early apoptotic and late apoptotic HT-29 cell
647 figures was determined using PI and Annexin V-FITC staining. The majority (68.4%,
648 right bottom quadrant) of HT-29 cells exhibited early apoptosis as indicated by the
649 limited nuclear fragmentation. Late apoptosis/necrosis was present in 28.9% (right
650 upper quadrant) of HT-29 cells with advanced nuclear fragmentation and limited
651 staining, while the frequency of normal cells dropped to 2.6% as observed in the left
652 bottom quadrant of the flow cytometry screen (n = 6).

653 **Figure 8.** Caspase-3, caspase-8, caspase-9 and caspase-7 mRNA expression analysis
654 following treatment of HT-29 cells with LFWGE (0.4 mg/mL, 0.8 mg/mL) for 48 h.
655 The differences in expression after treatment with the various concentrations of
656 LFWGE were calculated using the ANOVA method. n = 6.

657 **Figure 9.** LFWGE activation of caspase-3 in HT-29 cells. HT-29 cells were treated in
658 medium with 0.4, 0.8, and 1.5 mg/mL of LFWGE for 48 h, and then caspase-3
659 activity was estimated. Values are presented as the mean ±SD, and expressed as fold
660 increase over the pretreatment level (experimental/control).n = 6.

Tables

Table 1. Main composition (% Dry-Weight) of LFWGE and WGE

composition	WGE	LFWGE
2, 6-dimethoxybenzoquinone (μg/g)	33.85±2.85	181.10±4.56
protein (%, w/w)	32.90±0.57	34.36±0.23
total phenols (mg gallic acid /g)	20.81±0.11	28.48±0.16
total sugar (%, w/w)	43.05±1.23	33.04±1.03
lactic acid (mg/g)	0	27.7±1.2

The results are expressed as the mean (±SD), n = 3.

Table 2. Inhibition effect of the fraction on the proliferation of HT-29 cells

concentration（μg/mL）	inhibition（%）
control	
0	--
2,6-dimethoxy benzoquinone	
100	78.58±5.40
50	70.54±3.32
25	69.57±4.72
12.5	35.38±2.71
protein peak1	
200	88.6±2.85
100	56.8±2.64
50	51.0±1.98
25	28.4±1.25
protein peak2	
200	0.9±0.14
100	2.3±0.045
50	-1.2±0.012
25	1.1±0.044
sugar	
4000	2.6±0.15
2000	0.5±0.10
1000	9.9±0.36
500	10.01±0.15
250	7.5±0.14
lactic acid	
100	5.40±0.58
50	2.70±0.88
25	1.30±0.45

The results are expressed as the mean (±SD), n = 6.

Table 3. Identification of the proteins of Triticum aestivum in peak1

proteins	number(s) and sequence(s) of corresponding peptide(s)	genbank amino acid no.
peroxidase1	7 peptides: 33-42, rglsfdfyrr; 61-72, kdiglaagllrl; 126-142, rgavvscadilalaard; 167-190, rqdvlsdlpapssnvpsllallrp; 218-235, rlf prpdptinpp flarl; 253-270, rtpnvfdnqyyvdlvnre; 268-292, reglfvsdqdlftnditrpiverf	AAM88383.1 （GI:22001285）
crystal structure of the triticum aestivum xylanase inhibitor-i in complex with aspergillus niger xylanase-i.	5peptides: 135-160, rgstgvaglansglalpaqvasaqkv; 219-238, rvpvpegalatggvmlstrl; 271-287, raveavapfgvcydtkt; 342-365, rapavilggaqmedfvldfdmekk; 366-381, rlgfs rlphftgcggl	1T6G (GI:55669877)
xylanase inhibitor 725ACC	4 peptides: 156-181, rgstg vagladsglalpaqvasaqkv; 266-279, rdvyrplvdaftka; 364-387, rapavilggaqmedfvl dfdmekk; 393-403, rlphftgcggl	ABU55392.1 GI:156186243
unnamed protein product	4 peptides: 55-66, reiaiaaglvri; 132-161, raaivasgg ptfpvplgnldslapasqdkv; 182-202, rglgdvadlvalsgahtigrs; 232-251, rlqnldvvtpdlfdngyyka	CBC68269.1 GI:257651497
serpin	4 peptides: 32-62, ksaasnaafspvslhsalsllaagagsatrd; 171-182, klvlanalyfkg; 289-302, kisfgieasdllkc; 328-355, rvssvfhqafvevneqgteaaastaikm	ACN59483.1 GI:224589266
low molecular weight glutenin subunit	2 peptides: 209-228, kvflqqqcspvampqslars; 260-276, raiiysivlqeqqqvrg	ACA63852.1 GI:169666907
xylanase inhibitor, partial	2 peptides: 37-51, rsdvyrpfitafdra; 57-73, kvaavapfelcydsskl	ACO55043.1 GI:226427708
endochitinase	2 peptides: 207-230, raigtdllnnpdlvatdatvsfkt; 264-284, rvpgygvitniingglecgrg	CAA53626.1 GI:416029
LMW glutenin pGM107	2 peptides: 184-203, kvflqqqcspvampqslars; 310-325, rilptmcsvnvplyrt	AAS66085.1 GI:45477539
class II chitinase	3 peptides: 78-111, revaaflaqtshettggwatapdgafawgycfkq; 153-176, raigvdllsnpdlvatdptvsfkt; 244-266, rycdilgvgygdnldcynqrpfa	AAX83262.1 GI:62465514
0.19 dimeric alpha-amylase inhibitor, partial	2 peptides: 39-54, rdccqqlahisewcrc; 100-117, rlpivvdasgdgayvckd	AAV39514.1 GI:54778501
putative avenin-like a precursor	1peptides: 91-125, rcqavcsvaqiimrqqqgqsfgqpqqqvpveimrm	CAJ32654.1 GI:89143120
recname: Full=Cytochrome C.	1peptides: 99-109, radliaylkka	P00068.1 GI:118040
monomeric alpha-amylase	1peptides:	ABO45971.1

inhibitor, partial	1-15, sgpwswcdpatgykv	GI:134034581
recname:	2peptides:	Q9ST57.1
Full=Serpin-Z2A; AltName: Full=TriaeZ2a; AltName:Full=WSZ2a	171-182, rlvlgnalyfkg; 288-300, kisfgieasdllk	GI:75313847
Bp2A protein, partial	1peptides:	ABO40343.1
	198-208, kmkaleiaert	GI:133872552
root peroxidase	1peptides:	ACF70704.1
	110-125, kqtvscadiltvaard	GI:194425589
R2R3 MYB transcriptional	1peptides:	AEG64799.1
factor	39-49, slpsaagllrc	GI:334145749
BAX.2	1peptides:	ACN81638.1
	19-42, rlprpyhgggpe pddgvlllgrrg	GI:225193978
alternative oxidase	1peptides:	BAB88646.1
	64-91, kdnvastaaataeamqaakagavqaake	GI:19912727
histone deacetylase	1peptides:	ABG43091.1
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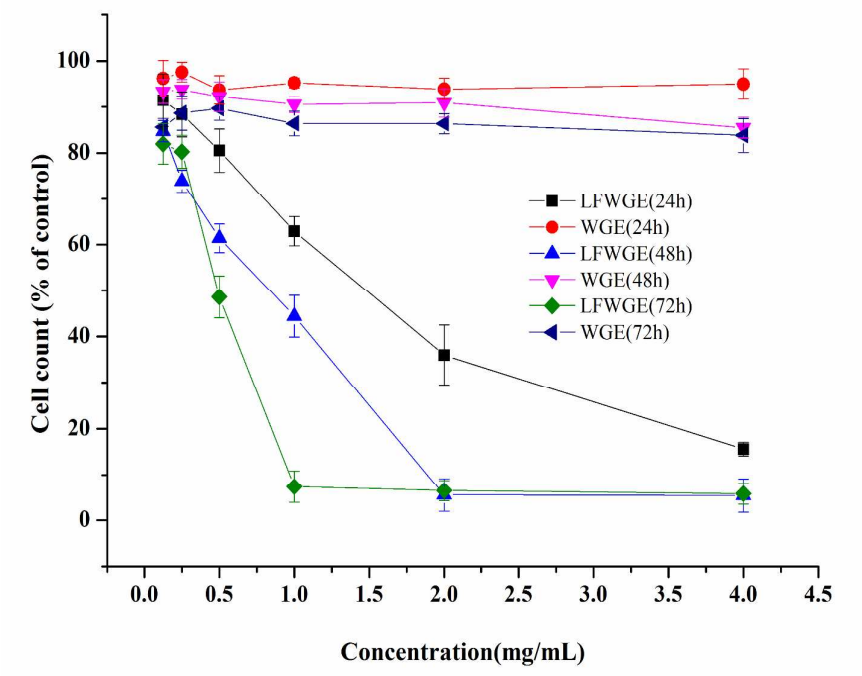


Figure 1

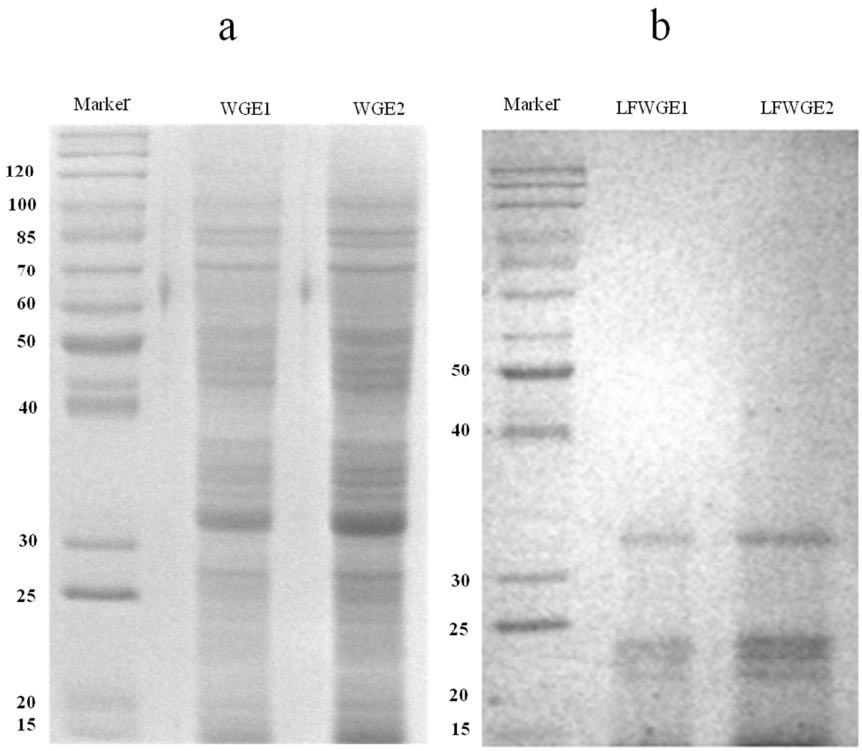


Figure 2

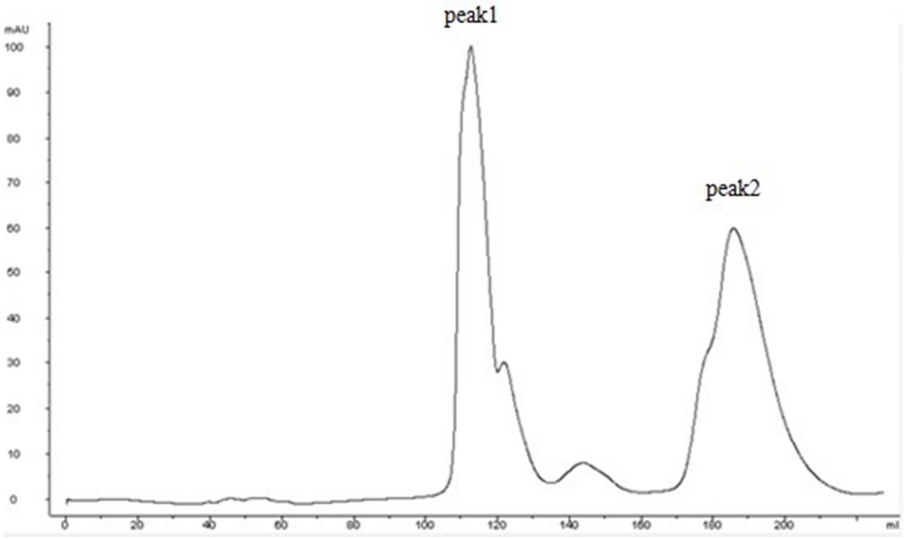


Figure 3

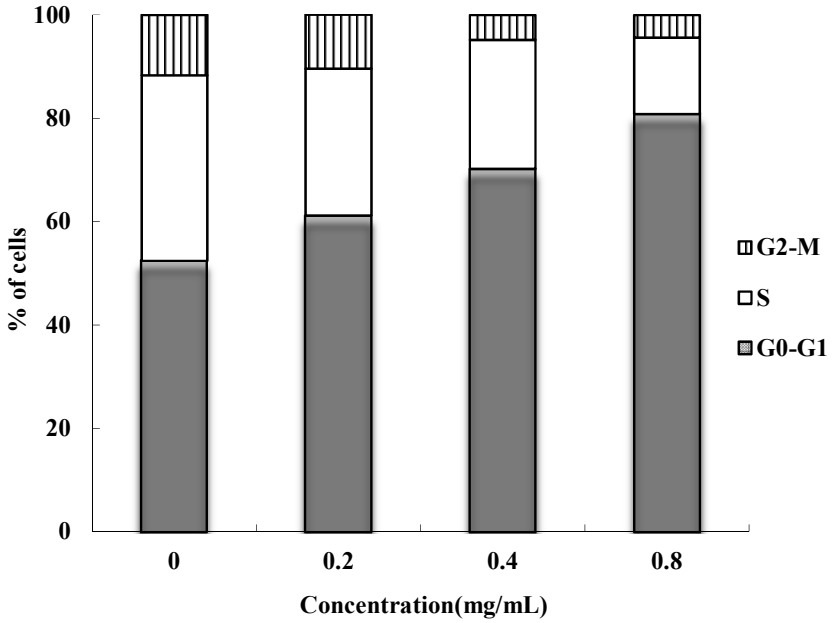


Figure 4

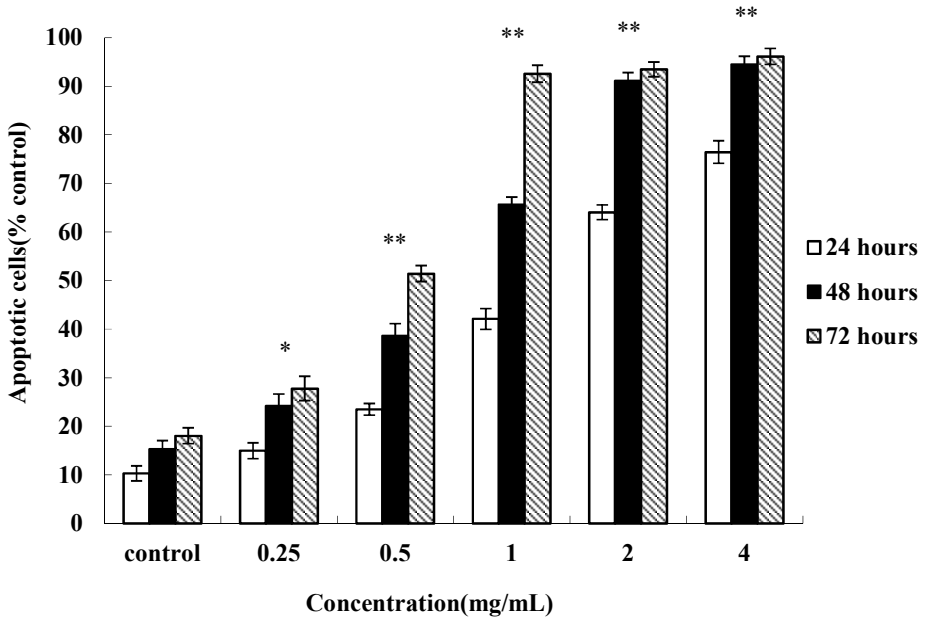
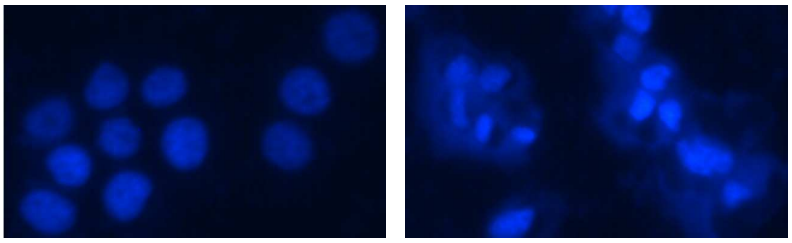


Figure 5



a **b**

Figure 6

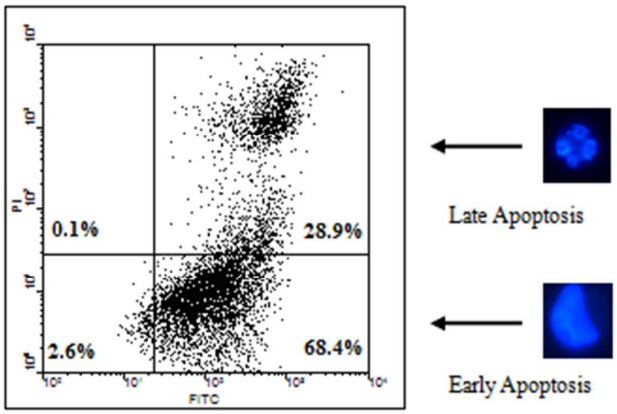


Figure 7

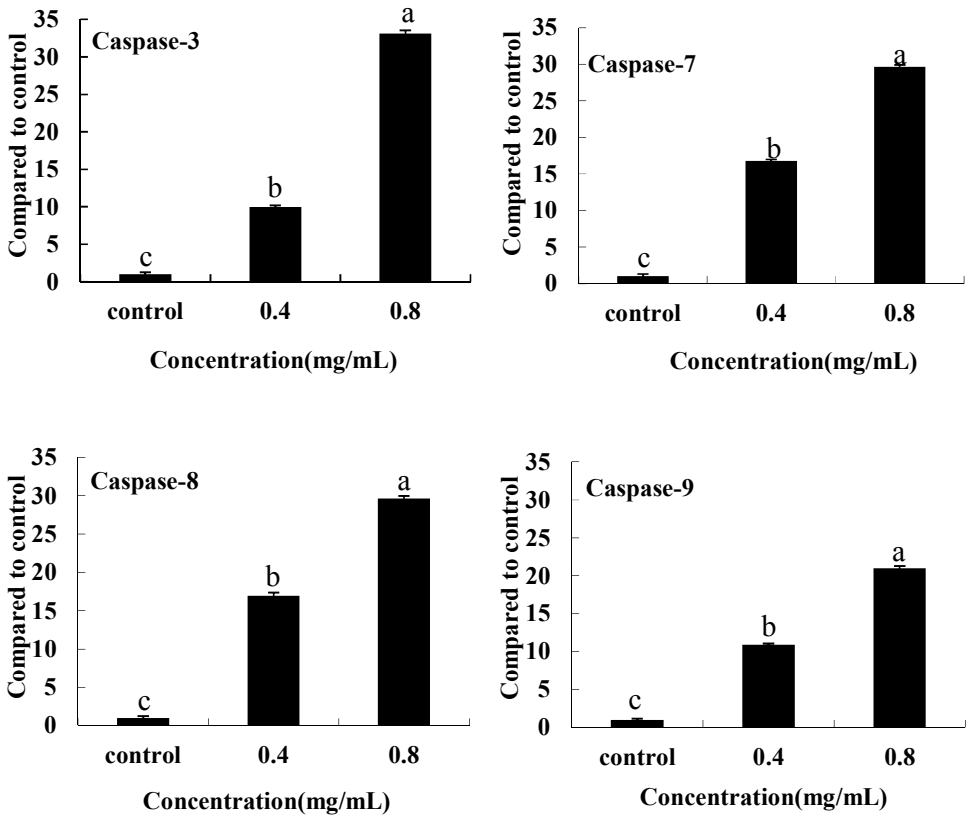


Figure 8

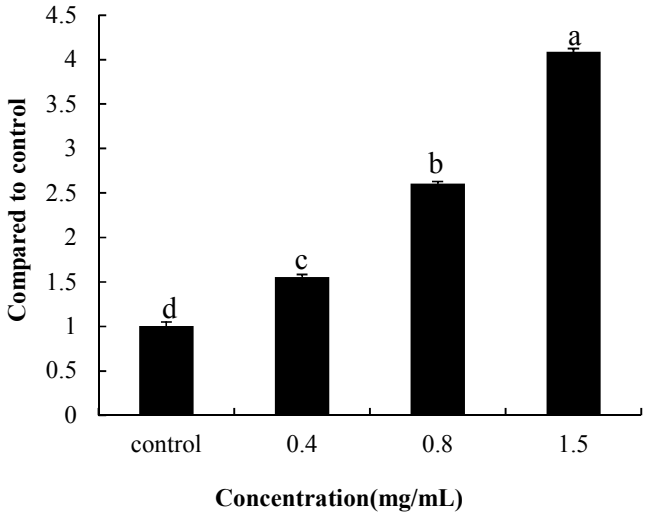


Figure 9

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