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4-Vinylguaiacol, an Active Metabolite of Ferulic Acid by Enteric Microbiota and Probiotics, Possesses Significant Activities against Drug-Resistant Human Colorectal Cancer Cells

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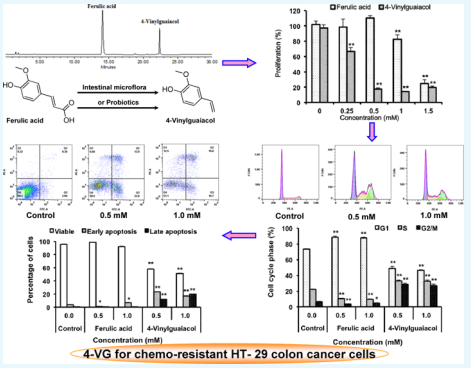
ABSTRACT: Ferulic acid, a hydroxycinnamic acid, is abundant in vegetables, grains, and medicinal plants. Emerging evidence suggests that ferulic acid may exert beneficial effects against colorectal cancer. However, the anticancer activity of ferulic acid is relatively low, and its metabolism after oral administration is largely unknown. In this study, mimicking the enteric environment, human intestinal microflora and commercial probiotics were used to metabolize ferulic acid to its metabolites, and their anticancer activities were evaluated. Ferulic acid can be biotransformed to 4-vinylguaiacol (2-methoxy-4-vinylphenol), and the contents of ferulic acid and 4-vinylguaiacol in bio-transformed extracts were determined by high-performance liquid chromatography (HPLC). Using the chemotherapy-sensitive cell line HCT-116 and the chemo-resistant cell line HT-29, the cell proliferation was determined by the modified trichrome stain assay. The cell cycle and induction of apoptosis were assayed using flow cytometry. HPLC data showed that there was a marked transformation from ferulic acid to 4-vinylguaiacol, and the conversion rates of intestinal microflora and four probiotics were from 1.3 to 36.8%. Both ferulic acid and 4-vinylguaiacol possessed dose- and time-related anticancer activities on the two cell lines, while 4-vinylguaiacol showed more potent effects than ferulic acid. Interestingly, 4-vinylguaiacol exhibited significantly higher antiproliferative effects on the HT-29 cell line than that on HCT-116. The IC50 of the metabolite 4-vinylguaiacol on HT-29 cells was 350 μM, 3.7-fold higher than its parent compound. The potential of cancer cell growth inhibition of 4-vinylguaiacol was mediated by cell cycle arrest at the G1 phase and induction of apoptosis. Data from this study indicate that the oral administration of ferulic acid offers a promising approach to increase its anticancer activity through gut microbial conversion to 4-vinylguaiacol, and the biotransformation could also be achieved by selected commercial probiotics. 4-Vinylguaiacol is a potential anticancer metabolite from ferulic acid for chemotherapy-resistant colon cancer cells.



1. INTRODUCTION

Cancer, which is one of the leading causes of death in the world, is a multi-step process occurring over an extended time frame, thus there are several possible stages at which the process halted, slowed down, or even reversed.[1,2] The clinical cancer management involves diverse conventional modalities including surgery, radiation, and chemotherapy.[3] A number of exogenous chemical compounds have been tested for possible chemoprevention activity to prevent, inhibit, or reverse the process of carcinogenesis.[4,5] These include dietary constituents, micronutrients, trace elements, and some pharmaceuticals.[6] In the past 30 years, nearly 80% of approved anticancer drugs were derived from natural compounds.[7] Recently, much attention has been focused on identifying phytochemicals, particularly those included in our diet, which possess the ability to interfere with carcinogenic and mutagenic processes.[1]

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies in the world, making up approximately 10% of all cancer cases in both men and women.[8] In the United States, it is estimated that there will be 147,950 new cases and 53,200 deaths from CRC in 2020, indicating the inadequacy of currently available treatment modalities.[9] However, even though the response rate to current systemic chemotherapies can reach up to 50%, drug resistance reportedly develops in nearly all patients with CRC and limits the therapeutic efficacies of anticancer agents and finally leads to chemotherapeutic failure.[10]

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Table 1. Linear Regression Data, LOD, and LOQ for Ferulic Acid and 4-Vinylguaiacol

analyte	compound	regression equation	R^2	test range (μg)	LOD (ng)	LOQ (ng)
1	ferulic acid	$Y = 1666140X + 10012$	0.9993	0.021−4.267	5.3	10.7
2	4-vinylguaiacol	$Y = 1187402X + 13737$	0.9995	0.019−3.800	4.7	9.5

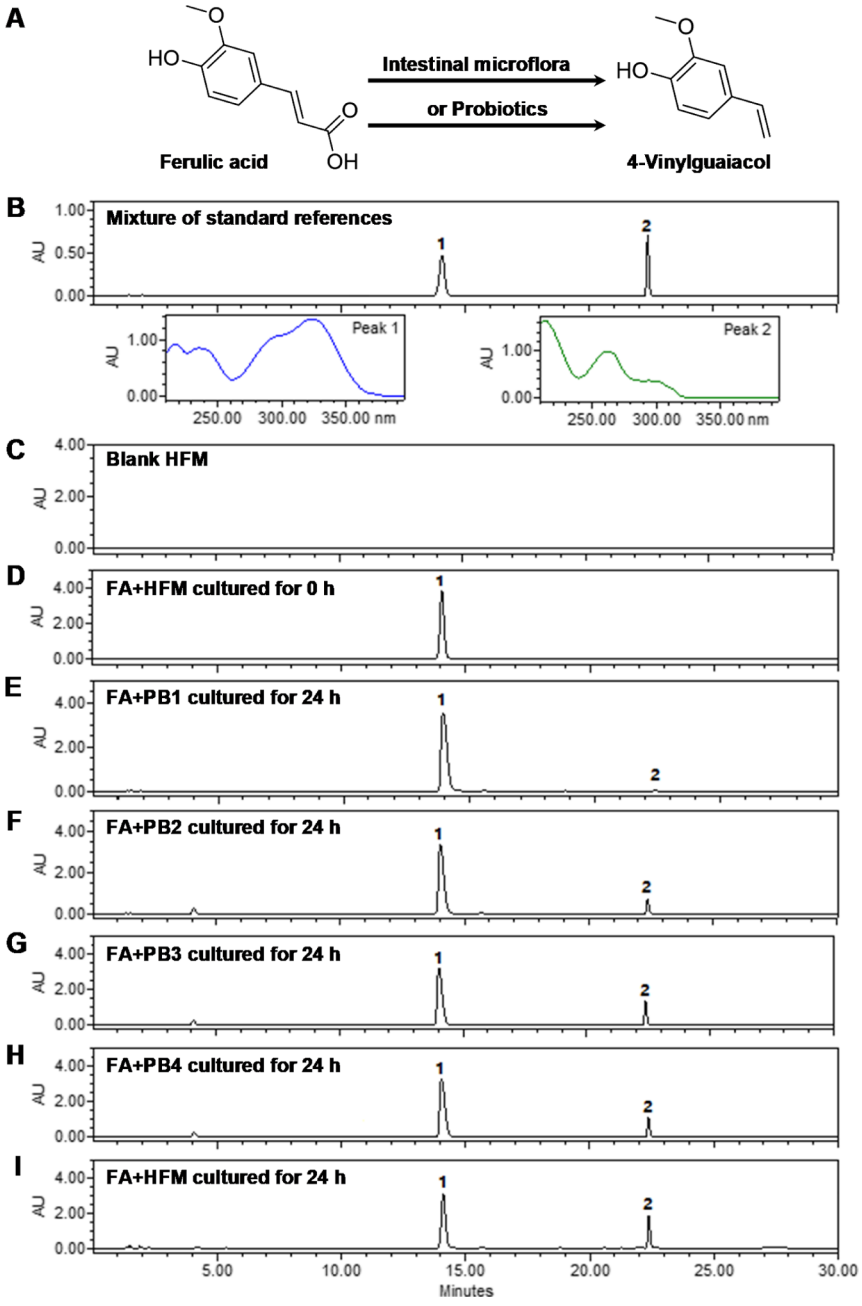

Figure 1. HPLC analysis of ferulic acid and its metabolite 4-vinylguaiacol. (A) Human intestinal microflora or probiotics bio-transformation from ferulic acid to 4-vinylguaiacol with their chemical structures displayed. (B) HPLC chromatogram of mixture standard references recorded at 272 nm and their UV spectra (200−400 nm). (C) Chromatogram of vehicle control. (D) Chromatogram of ferulic acid and 4-vinylguaiacol at 0 h. (E−H) Chromatograms of ferulic acid treated with four different commercial probiotics (PB) 1−4. See Figure 2 for detailed information of these PBs. (I) HPLC chromatogram of ferulic acid treated with HFM. Peak 1, ferulic acid; peak 2, 4-vinylguaiacol.

Controlling the growth of drug-resistant CRC cells becomes a big challenge for the treatment of CRC.[8] Therefore, it is necessary and meaningful to find new compounds extracted from botanicals or functional foods in treating CRC, especially the drug-resistant CRC. The two cell lines used in this study varied in p53 expression. HCT-116 is a p53 wild type, whereas HT-29 cells contain a p53 mutation. Cancer cells with p53 mutations are resistant to many chemotherapeutic agents. Thus, the effects of agents on those two types of cell lines reflect the treatment responses of two types of CRC cells, that is, chemo-sensitive and chemo-resistant cells.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid), a hydroxycinnamic acid, is abundant in vegetables and grains, such as onions, beans, flaxseeds, corn, wheat, and rice bran.[11] Ferulic

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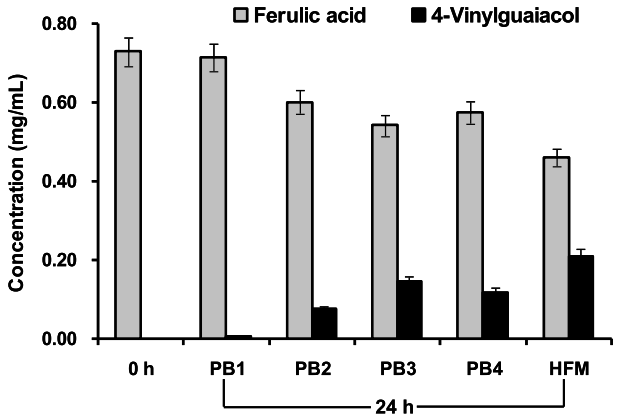

Probiotics	Strains
PB1	10 strains: *Lactobacillus acidophilus* (La-14), *L. casei* (Lc-11), *L.lactis* (Ll-23), *L. paracasei* (Lpc-37), *L. plantarum* (Lp-115), *L. rhamnosus* (Lr-32), *Bifidobacterium breve* (Bb-03), *B. bifidum* (Bb-06), *B. lactis* (Bl-04), *B. longum* (Bl-05).
PB2	10 strains: La-14, Lc-11, Lpc-37, Lp-115, Lr-32, *L. salivarius* (Ls-33), Bb-03, Bl-04, Bl-05, *Streptococcus thermophilus* (St-21).
PB3	10 strains: La-14, *L. bulgaricus* (Lb-87), Lc-11, Lpc-37 , Lp-115, Ls-33, *B. bifidum* (Bb-02) , Bb-03, Bl-04, Bl-05.
PB4	4 strains: La-14, Lpc-37, Lp-115, Bl-04.

Figure 2. The contents of ferulic acid and its metabolite 4-vinylguaiacol treated with four different commercial probiotics (PB1−PB4) and HFM (left side). Strains in these probiotics are shown on the right side. PB1 obtained from Intelligent Labs; PB2 from NOW Foods; PB3 from Physician's choice; and PB4 from BioSchwartz LLC.

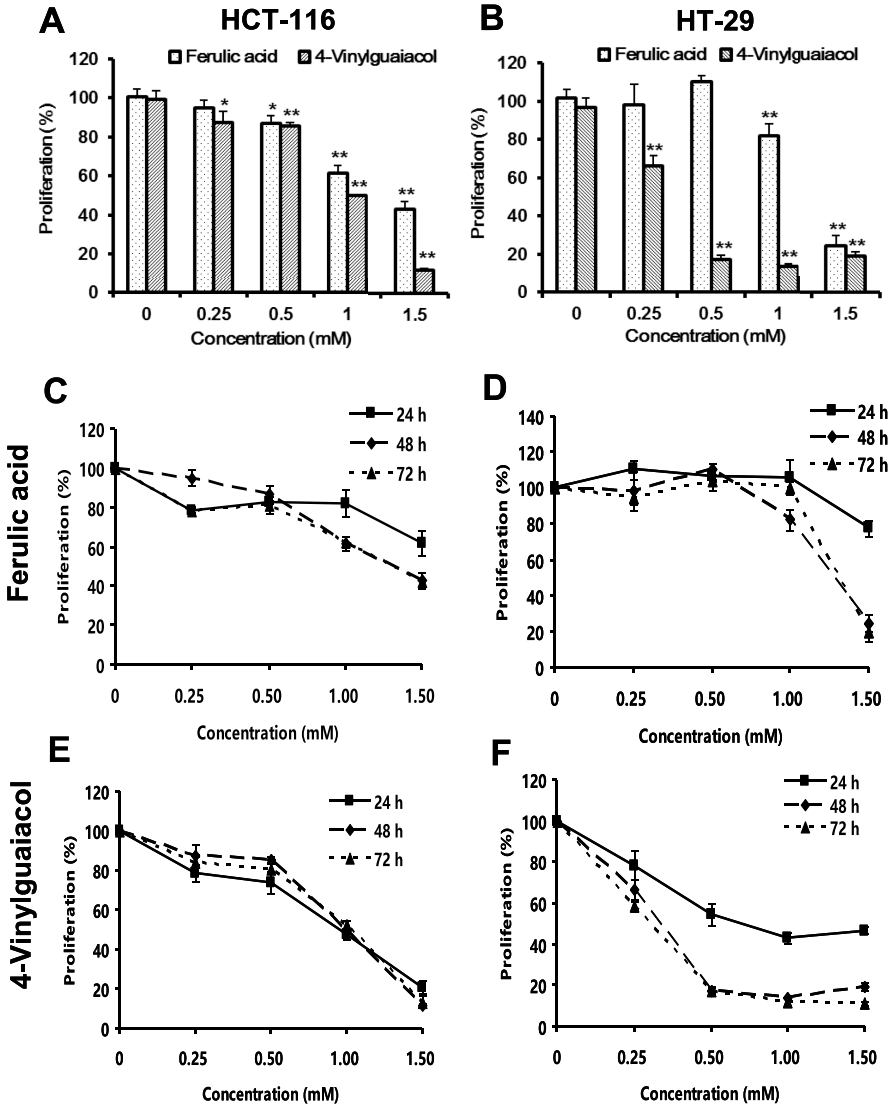

Figure 3. Effects of ferulic acid and 4-vinylguaiacol on cell proliferation in HCT-116 and HT-29 human colorectal cancer cell lines. Concentration-related anti-proliferative effects of ferulic acid and 4-vinylguaiacol on HCT-116 cells (A) and HT-29 cells (B) for 48 h. Time-related anti-proliferative effects of ferulic acid on HCT-116 cells (C) and HT-29 cells (D) and the counterpart of 4-vinylguaiacol on HCT-116 cells (E) and HT-29 cells (F). *$P < 0.05$ and **$P < 0.01$ vs control.

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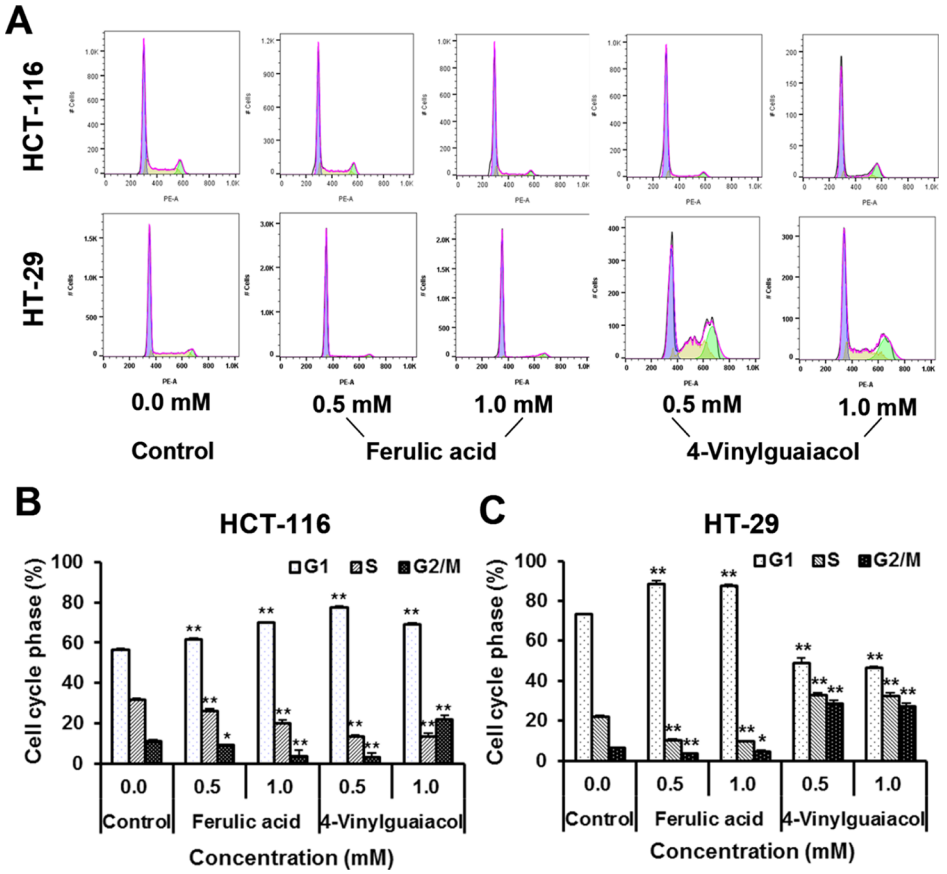

Figure 4. Cell cycle analysis of HCT-116 and HT-29 cells using flow cytometry following treatment with ferulic acid or 4-vinylguaiacol on HCT-116 cells and HT-29 cells for 48 h stained with PI. (A) Typical cell cycle profiles and (B−C) interpretation of data. Data are presented as mean ± standard error (SE) of triplicate experiments. *P < 0.05 and **P < 0.01, vs control.

acid has also been found in many Chinese herbal medicines, such as *Ligusticum chuanxiong*, *Angelica sinensis*, *Cimicifuga heracleifolia*, and *Ferula assafoetida*.[12] Several biological activities of ferulic acid have been reported such as antioxidant, anti-inflammatory, anti-cancer, anti-apoptotic, antidiabetic, and hepatoprotective.[13,14] Emerging evidence suggests that ferulic acid may exert beneficial effects on CRC.[11,15] However, the anticancer activity of ferulic acid is comparatively low.[16,17] Our previous studies showed that the anticancer activity of some phytochemicals could be enhanced by biotransformation of the human enteric microbiome.[18,19] Although ferulic acid could be converted to 4-vinylguaiacol by certain strains of yeast,[20] the metabolism of this compound by the human gut microbiome and the anticancer activity of its metabolites are largely unknown.

Probiotics commonly refer to viable microorganisms that originate from the gut and have beneficial health impacts on the host.[21] Due to the significant role of probiotics in enhancing the gut health and overall human well-being, the demand for probiotic products has increased exponentially over the recent years.[22−24] Commercial available probiotics often contain mixtures of two or more individual species, such as *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus* spp.[25] It would be interesting to know whether ferulic acid could be converted to 4-vinylguaiacol by probiotics for potential CRC management.

In this project, the biotransformation of ferulic acid to 4-vinylguaiacol by human intestinal microflora and selected probiotics was measured and compared by high-performance liquid chromatography (HPLC), and then the anti-CRC activities of ferulic acid and 4-vinylguaiacol were investigated. In addition to the commonly studied human CRC cell line HCT-116, the drug-resistant CRC cell line HT-29 was used to evaluate whether these two compounds possess anti-CRC effect against the chemo-resistant cell line. The related mechanisms of actions were explored.

2. RESULTS

2.1. Ferulic Acid Metabolism by Enteric Microbiome and Commercial Probiotics. HPLC analysis was used to detect the levels of ferulic acid and its metabolite, 4-vinylguaiacol, after the biotransformation of human enteric microbiome and commercial probiotics. Table 1 shows the regression equation and limits of detection and quantitation (LOD and LOQ, respectively) of ferulic acid and 4-vinylguaiacol, which were considered to be satisfactory for subsequent analysis of all the samples.

Figure 1A shows the chemical structures of ferulic acid and its bio-transformed metabolite 4-vinylguaiacol. The HPLC chromatogram of mixture standard of ferulic acid and 4-vinylguaiacol is shown in Figure 1B. The peaks of ferulic acid (peak 1) and 4-vinylguaiacol (peak 2) are separated very well. Ultraviolet spectroscopy of ferulic acid and 4-vinylguaiacol is also shown in Figure 1B. To determine whether fecal compounds influence ferulic acid analysis, we assayed the vehicle fecal sample. No obvious peak was observed in the chromatogram of the fecal sample (Figure 1C). Figure 1D shows the chromatogram of ferulic acid cultured with human

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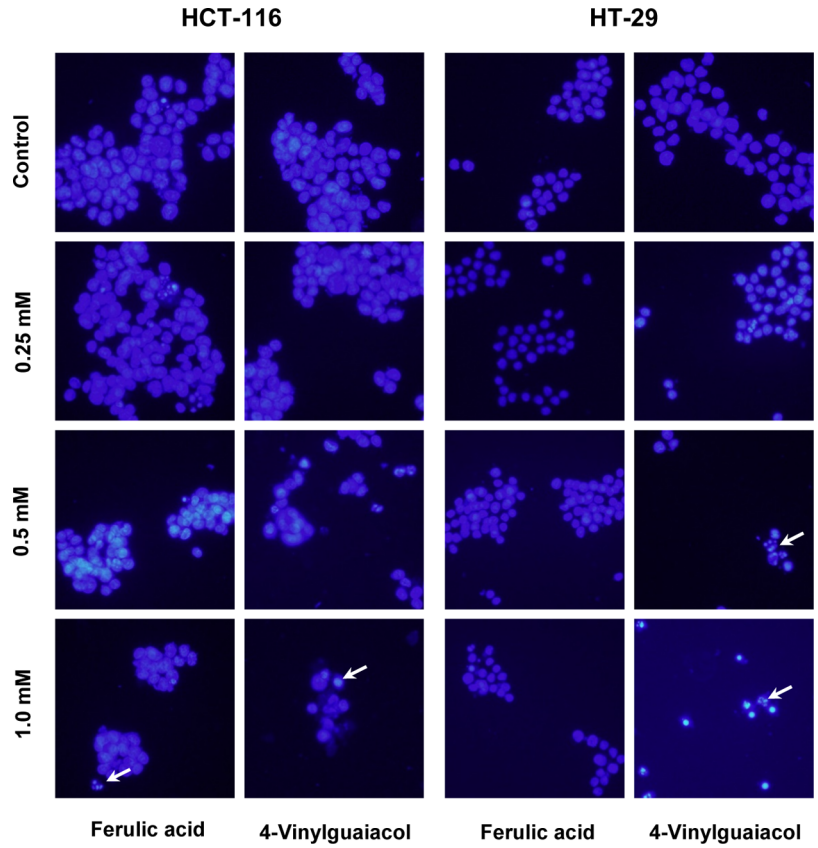

Figure 5. Ferulic acid- and 4-vinylguaiacol-induced apoptosis on HCT-116 and HT-29 human colorectal cancer cells. HCT-116 and HT-29 cells were treated with different concentrations of ferulic acid or 4-vinylguaiacol for 48 h. The cells were stained with a DNA specific dye, Hoechst 33258, and imaged at ×20 magnification in a fluorescence microscope. Apoptotic cells are indicated with arrows.

enteric microbiome for 0 h. Thus, compounds from the fecal sample did not influence ferulic acid determination. Figure 1E−H shows the chromatograms of ferulic acid cultured with four different commercial probiotics for 24 h. There were marked transformations from ferulic acid to 4-vinylguaiacol. When ferulic acid was cultured with human enteric microbiome for 24 h, compared to untransformed ferulic acid, the ferulic acid peak was significantly reduced (Figure 1I).

As shown in Figure 2, about 36.8% of ferulic acid was converted to 4-vinylguaiacol after being cultured with human fecal microbiome (HFM) for 24 h. Our data also show that the commercial probiotics also transformed ferulic acid to 4-vinylguaiacol with different rates of transformation from 1.3 to 25.6% (PB1: 1.3%, PB2: 13.9%, PB3: 25.6%, and PB4: 21.0%). The strains of four commercial probiotics are shown in Figure 2.

2.2. Antiproliferative Effects of Ferulic Acid and 4-Vinylguaiacol. Cell proliferation plays an important role in the initiation and promotion steps of carcinogenesis.[26] Therefore, control of cell proliferation is important for cancer prevention. We evaluated the effects of ferulic acid and 4-vinylguaiacol on cell proliferation in HCT-116 and HT-29 human CRC cell lines.

As shown in Figure 3A,B, while 48 h treatment with ferulic acid inhibited cancer cell growth in relatively high concentrations, 4-vinylguaiacol caused much stronger growth suppression in both HCT-116 and HT-29 CRC cell lines than in ferulic acid. At the concentration of 1.0 mM, ferulic acid showed an anti-proliferative effect of 38.4 ± 3.7% on HCT-116 cells ($P < 0.05$ vs control), while no significant effects were observed on HT-29 cells. At the same concentration (1.0 mM), 4-vinylguaiacol inhibited cancer cell growth by 50.0 ± 3.4% in HCT-116 cells and 85.9 ± 3.9% in HT-29 cells, respectively (both $P < 0.01$ vs control). Compared to ferulic acid, 4-vinylguaiacol showed more significant anti-proliferative effects in both HCT-116 and HT29 cells with IC_{50} values of 1.0 and 0.35 mM, while the IC_{50} values of ferulic acid were about 1.3 and 1.3 mM, respectively. Among the two cell lines, 4-vinylguaiacol showed the most significant anti-proliferative effects in HT-29 cells with an IC_{50} value of 0.35 mM, while the IC_{50} value of ferulic acid was about 1.3 mM. Figure 3C−F shows the time-associated anti-proliferative effects of ferulic acid on HCT-116 cells (Figure 3C) and HT-29 cells (Figure 3D) and the counterpart of 4-vinylguaiacol on HCT-116 cells (Figure 3E) and HT-29 cells (Figure 3F). Our data suggested that both ferulic acid and 4-vinylguaiacol showed dose- and time-related anticancer activities on two cell lines, while 4-vinylguaiacol showed more potent effects than ferulic acid. Interestingly, 4-vinylguaiacol showed significantly higher anti-proliferative effects on the drug-resistant human CRC HT-29 cell line than that on HCT-116. The IC50 for the metabolite 4-vinylguaiacol on HT-29 cells was 350 μM, which is 3.7-fold stronger than its parent compound ferulic acid.

2.3. Effects of Ferulic Acid and 4-Vinylguaiacol on Cell Cycle. Antiproliferative evaluation suggested that ferulic acid and 4-vinylguaiacol were active in inhibiting both HCT-116 and HT-29 human CRC cell growth. To explore whether this was because of cell cycle arrest at a specific phase, the cell cycle profile was assayed using flow cytometry.

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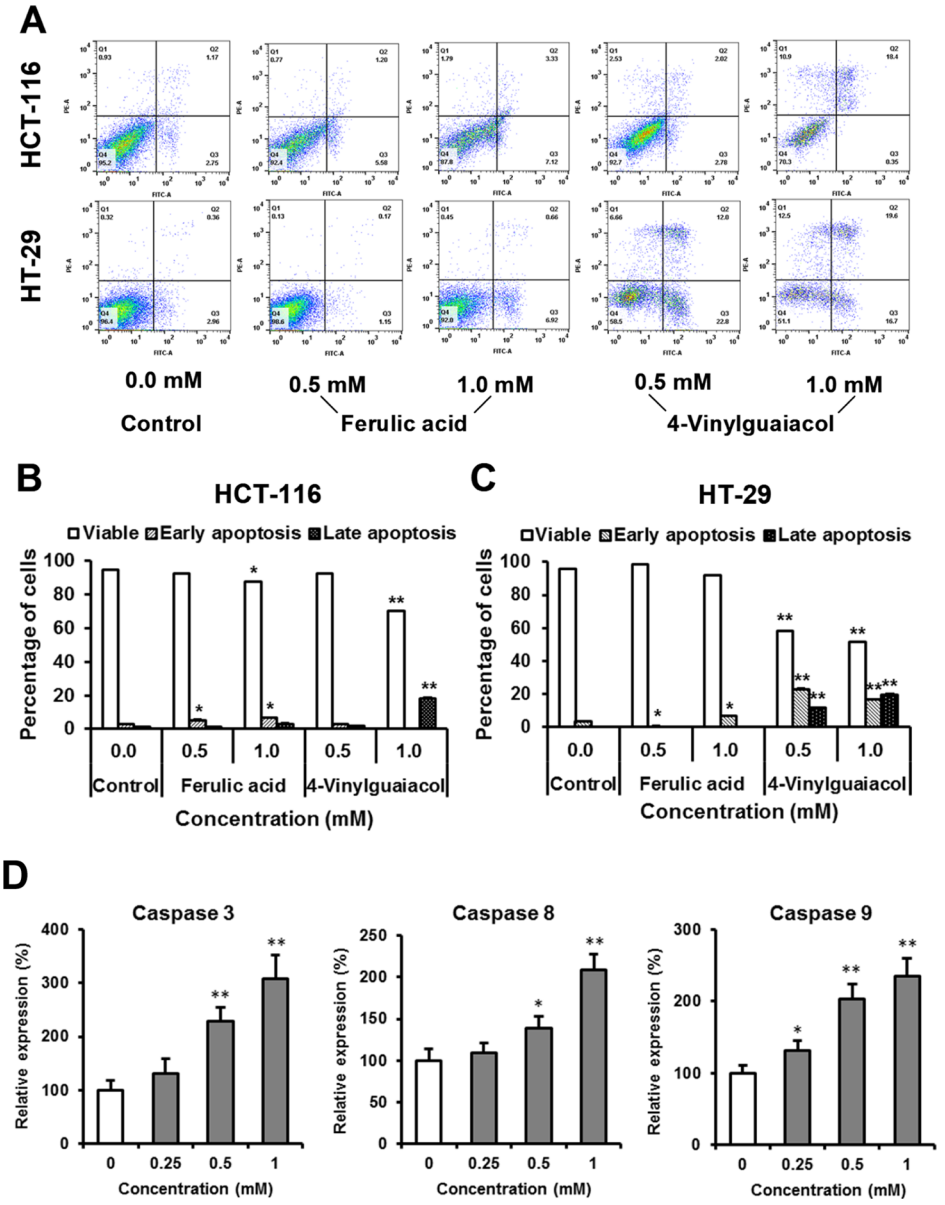

Figure 6. Apoptosis analysis using flow cytometry following staining with annexin V-fluorescein isothiocyanate/PI. HCT-116 and HT-29 cells treated with ferulic acid and 4-vinylguaiacol at 0.5 and 1.0 mM for 48 h. (A) Representative scatter plots of PI (*y*-axis) vs annexin V (*x*-axis). (B,C) Percentage of viable, early apoptotic, and late apoptotic cells. (D) Effects of 4-vinylguaiacol on caspase 3, 8, and 9 activities in HT-29 cells. Data are presented as mean ± SE of triplicate experiments. *$P < 0.05$ and **$P < 0.01$ vs control.

As shown in Figure 4, compared to the control, the effects of ferulic acid and 4-vinylguaiacol on the cell cycle profile were observed at concentrations as low as 0.5 mM. As shown in Figure 4B, treatment of HCT-116 cells with 0.5 and 1.0 mM ferulic acid for 48 h decreased the S phase to 25.9 and 19.6%, respectively, compared to 31.6% in vehicle-treated cells while increasing the G1 phase to 61.5 and 69.7%, respectively, compared to 56.3% in vehicle-treated cells (both $P < 0.01$). Treatment of HCT-116 cells with 0.5 and 1.0 mM 4-vinylguaiacol for 48 h decreased the S phase to 13.3 and 13.2%, respectively, compared to 31.6% in vehicle-treated cells, while increasing the G1 phase to 77.1, and 68.8%, respectively, compared to 56.3% in vehicle-treated cells (both $P < 0.01$). As shown in Figure 4C, treatment of HT-29 cells with 0.5 and 1.0 mM ferulic acid for 48 h increased the G1 phase to 88.6 and 87.6%, compared to 73.4% in vehicle-treated cells, while decreasing the S phase to 10.4 and 9.6%, compared to 22.2% in vehicle-treated cells ($P < 0.01$). Treatment of HT-29 cells with 0.5 and 1.0 mM 4-vinylguaiacol for 48 h decreased the G1 phase to 49.0 and 46.3%, respectively, compared to 73.4% in vehicle-treated cells, while increasing the S phase to 32.9 and 32.6%, respectively, compared to 22.2% in vehicle-treated cells (both $P < 0.01$).

We observed that ferulic acid significantly decreased the cancer cell proportion in S and G2/M phases and increased the proportion of G1 phase in both cancer cell lines. On the other hand, for the HT-29 cell line, 4-vinylguaiacol significantly decreased the cell proportion in the G1 phase and increased the cell proportion in S and G2/M phases. These results suggested that the metabolite 4-vinylguaiacol significantly induced S and G2/M phase cell cycle arrest in the chemo-resistant HT-29 cells.

2.4. Effects of Ferulic Acid and 4-Vinylguaiacol on Apoptosis. There are two types of cell death in biological

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systems, namely, necrosis and apoptosis. Apoptosis, which is a highly regulatory process of programed cell death, is considered to be an important mechanism in the inhibition of cancer cells, and many cancer chemotherapeutic drugs are strong inducers of apoptosis against cancer cells.[27] Apoptotic cancer cells can be observed with a fluorescence microscope after they are stained with Hoechst 33258. To test whether or not the decrease in cell viability observed after treatment with ferulic acid and 4-vinylguaiacol was due to apoptosis, HCT-116 and HT-29 cells were stained with Hoechst 33258 dye after exposure to the compounds for 48 h. The dye stains condensed the chromatin of apoptotic cells more brightly than the chromatin of normal cells (Figure 5).

Induction of apoptosis was also confirmed by flow cytometry after staining with annexin V and propidium iodide (PI). Annexin V can be detected in both early and late stages of apoptosis, whereas PI-stained cells were only in late apoptosis or necrosis. Early apoptotic cells were positive for annexin V and negative for PI (lower right quadrant); late apoptotic cells stained for both annexin V and PI (upper right quadrant).

As shown in Figure 6B, following treatment with 0.5 and 1.0 mM ferulic acid for 48 h, compared to the control (2.9%), the percentage of early apoptotic HCT-116 cells increased to 5.4 and 7.0%, respectively ($P < 0.05$ and $P < 0.01$). Upon treatment with 1.0 mM ferulic acid for 48 h, compared to the control (1.2%), the percentage of late apoptotic HCT-116 cells increased to 3.2% ($P < 0.05$). After treatment with 1.0 mM 4-vinylguaiacol for 48 h, the percentage of late apoptotic HCT-116 cells increased to 18.0% ($P < 0.01$ vs control of 1.2%).

As shown in Figure 6C, following treatment with 1.0 mM ferulic acid for 48 h, the percentage of early apoptotic HT-29 cells increased to 6.8% ($P < 0.05$), compared to the control (3.8%). Following treatment with 0.5 and 1.0 mM 4-vinylguaiacol for 48 h, the percentage of early apoptotic HT-29 cells increased to 23.2 and 16.8%, respectively (both $P < 0.01$ vs control of 3.8%). Treatment with 0.5 and 1.0 mM 4-vinylguaiacol for 48 h increased the percentage of late apoptotic HT-29 cells to 12.0% and 19.8%, respectively (both $P < 0.01$ vs control of 0.4%). These data demonstrate that 4-vinylguaiacol significantly induced HCT-116 and HT-29 cell apoptosis, especially in the late stage of apoptosis. Meanwhile, more potent effects were observed on the chemo-resistant HT-29 cell line. Caspase 3, 8, and 9 data supported flow cytometry data that 4-vinylguaiacol induced caspase-dependent cell death (Figure 6D).

3. DISCUSSION

The human enteric microbiome plays an important role in gut homeostasis and overall health. The commensal gut microbiome protects the host by displacing harmful bacteria, competing with pathogens for nutrients, and producing antimicrobial factors.[28] These commensal bacteria also provide the host with structural functions, such as developing the immune system and reinforcing the mucosal barrier.[29] Furthermore, these bacteria metabolize dietary components and ferment non-digestible dietary foods resulting in the formation of short-chain fatty acids.[30] Ferulic acid is abundant in different kinds of food and herbal medicines, but its gut metabolism has not been reported yet. In this study, we examined the biotransformation of ferulic acid by human gut microbiota and probiotics using HPLC, and 4-vinylguaiacol has been identified as a main metabolite of ferulic acid. We also found that under the same culture conditions, the intestinal flora of healthy people has a desirable conversion rate of ferulic acid to 4-vinylguaiacol, which is higher than that of the four probiotic products we tested.

Natural compounds and their metabolites can be classified based on their hydrophilic or hydrophobic characteristics. Using a reverse-phase HPLC column, more hydrophilic compounds are eluted first. Then, compounds from hydrophilic to hydrophobic are gradient-eluted. Compared to ferulic acid, 4-vinylguaiacol is a hydrophobic compound, and a more hydrophobic metabolite has stronger affinity to the cell membrane and thus is more readily transported into the cell for biological activities compared to that of ferulic acid. Although there is no pharmacokinetic data available, we predict that 4-vinylguaiacol has higher biomedical effects compared to that of ferulic acid, and this will be evaluated in future *in vivo* studies.

The intestinal flora is affected by many factors such as diet, drugs, especially antibiotics, and diseases and has become a potential target for the prevention and treatment of many diseases.[31] Several studies have shown that gut microbial dysbiosis can be associated with the development of both intestinal and extra-intestinal disorders.[32] Cancer patients often have gut microbial dysbiosis due to the use of antibiotics, radiotherapy, chemotherapy, and so forth. Administering probiotics may help restore the balance of intestinal flora and help convert some food chemicals to potentially effective antitumor substances. The preventive and therapeutic role of probiotics in cancer has been established *via* several mechanisms including modulation of gut microbiota, enhancement of gut barrier functions, degradation of potential carcinogens, and enhancement of the immune system.[31] In this study, the preventive and therapeutic role of probiotics in human CRC might be established indirectly *via* transforming parent components to metabolites with significant anti-cancer effects. Although the probiotic product PB4 only contains four species of bacteria, the conversion rate of ferulic acid is higher than that of probiotics PB1 and PB2 (each with 10 species). Thus, the conversion rate of ferulic acid by probiotics is more likely related to type, rather than the number, of bacterial species. In future studies, it remains to be elucidated which bacteria in probiotics played the key role in the conversion of ferulic acid.

Drug resistance develops in nearly all CRC patients, and this limits the therapeutic efficacies of anticancer drugs and finally leads to chemotherapy failure. It is meaningful to find new compounds for the treatment of drug-resistant CRC, and the anticancer activities of ferulic acid have been reported.[11,15] However, ferulic acid only possesses limited anticancer activities. In the research on natural products, many previous studies employed primarily reductionist approaches in screening compounds for bioactivity, and different parent compounds were investigated. However, after oral administration, whether ferulic acid can be metabolized by the enteric microbiome and if its microbial metabolites lead to more powerful anti-CRC activities are largely unknown.

In this study, we investigated the biotransformation of ferulic acid by the human enteric microbiome and several probiotics. To compare the antiproliferative effects of ferulic acid and its metabolite 4-vinylguaiacol against CRC, we used a wild-type HCT-116 and the drug-resistant cancer cell line HT-29. Although the parent compound ferulic acid showed antiproliferative effects in both cancer cell lines, the IC50 is relatively high (>1.3 mM). Compared with ferulic acid, the

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metabolite 4-vinylguaiacol showed much stronger antiproliferative effects in both cell lines, especially in the drug-resistant cancer cell line HT-29 with an IC50 of 0.35 mM, which is 3.7-fold stronger than that of ferulic acid, suggesting that 4-vinylguaiacol is a potentially active anti-CRC metabolite.

The mechanisms of 4-vinylguaiacol on colon cancer chemoprevention are largely unclear. Inhibition of cell cycle progression and induction of apoptosis are important mechanisms mediating the effects of many anti-cancer agents. One of the important characteristics of malignant tumors is that the regulation of the cell cycle is out of control, causing cells to grow disorderly and lack differentiation. In addition, evasion of apoptosis, one of the hallmarks of human cancers, contributes to carcinogenesis and tumor progression, as well as drug resistance in cancer.[33]

In this study, we evaluated the effects of 4-vinylguaiacol on the cell cycle and apoptosis. 4-Vinylguaiacol can significantly arrest HT-29 cells in S and G2/M phases and markedly induce CRC cell apoptosis at concentrations as low as 0.5 mM. Furthermore, the antiproliferative potential of 4-vinylguaiacol on the chemo-resistant CRC cell line HT-29 is comparable to that of 5-fluorouracil (a first-line chemotherapeutic agent against CRC) when it is tested on HCT-116 cells, a chemo-sensitive CRC cell line.[34] Comparing the effects of 4-vinylguaiacol on the cell cycle and apoptosis, we showed that induction of apoptosis appeared to be a greater effect than cell cycle deceleration. This result suggests that the cancer cell growth-inhibitory effect of 4-vinylguaiacol was predominantly mediated by induction of apoptosis.

On comparing the structural differences and antiproliferative activities of ferulic acid and 4-vinylguaiacol, the elimination of a −COOH group in ferulic acid and the formation of a $-CH{=}CH_2$ group in 4-vinylguaiacol are likely to increase the metabolite's anticancer effects. The presence of a −COOH group reduces the hydrophobic character of ferulic acid and decreases its ability to permeate the cell membrane, while the presence of the $-CH{=}CH_2$ group increases the hydrophobic character of 4-vinylguaiacol and thus has a stronger affinity to the cancer cell membrane, so that it is more readily transported into cells to exert their biological activities.

4. CONCLUSIONS

In conclusion, we demonstrated for the first time, using the human enteric microbiome and probiotics, that the parent compound, ferulic acid, can be readily converted to its metabolite, 4-vinylguaiacol. Using a chemotherapy-sensitive CRC cell line HCT-116, and also a chemo-resistant CRC cell line HT-29, the antiproliferative activities of these two compounds were evaluated. The parent compound ferulic acid showed relatively low antiproliferative effects. After biotransformation by gut microbiome or commercial probiotics, the metabolite 4-vinylguaiacol showed significant anticancer effects, especially on drug-resistant CRC cells. These observations were further supported by our cell cycle and apoptotic analyses. Future *in vivo* experiments using CRC animal models should be conducted to support potential clinical utility of 4-vinylguaiacol against drug-resistant colorectal malignancies.

5. MATERIALS AND METHODS

5.1. Chemicals and Materials. All solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and of HPLC grade. Deionized water was supplied by a Millipore water purification system (Burlington, MA, USA). Ferulic acid and 4-vinylguaiacol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Structures of ferulic acid and 4-vinylguaiacol were characterized by HPLC retention times and UV spectra based on the published literature.[35,36] Ferulic acid and 4-vinylguaiacol were of analytical-reagent grade with purity of at least 99%, as confirmed by HPLC. The soybean-casein digest medium was obtained from Becton, Dickinson, and Company (Sparks, MD, USA). Probiotics were from Intelligent Labs (Sarasota, FL, USA) (PB1), NOW Foods (Bloomingdale, IL, USA) (PB2), Physician's Choice (Westminster, CO, USA) (PB3), and BioSchwartz LLC (Jackson, WY, USA) (PB4).

All cell culture plastic materials were obtained from Corning Incorporated (Corning, NY, USA) and Fisher Scientific (Pittsburgh, PA, USA). McCoy's 5A medium, trypsin, and phosphate-buffered saline (PBS) were obtained from Mediatech, Inc. (Manassas, VA, USA). Fetal bovine serum (FBS) and penicillin G/streptomycin solution were purchased from Life Technologies Corporation (Grand Island, NY, USA). The modified trichrome stain (MTS) assay kit (CellTiter 96 Aqueous Cell Proliferation Assay) was obtained from Promega Corporation (Madison, WI, USA). The fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit and the PI/RNase staining buffer were obtained from BD Biosciences (San Diego, CA, USA). Hoechst 33258, formaldehyde, and NP40 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3, 8, and 9 kits were obtained from BioVision (Mountain View, CA, USA).

5.2. Compound Biotransformation Using HFM and Commercial Probiotics. Human enteric microflora in fecal samples were obtained from three adult volunteers, who were non-smokers and had not consumed antibiotics for more than 3 months before the study. The samples were collected by the donors in plastic cups and were processed within 30 min of the passage. All three fecal samples were mixed, and an aliquot of 10 g of mixed feces was homogenized with 30 mL of physiological saline to obtain a fecal slurry. The slurry was filtered through cotton muslin to remove particulate materials. 1 mL of fecal slurry or probiotic solution was mixed with 14 mL of soybean-casein digest medium containing 15 mg of ferulic acid. They were anaerobically incubated at 37 °C for 24 h. Then, the reaction solution was acidified with 0.3 mL of 0.1 M HCl and then extracted with 15 mL of *n*-butanol. The *n*-butanol solution was dried under a nitrogen steam spray in a water bath (60 °C). Then the residue was dissolved in methanol. The methanol solution was centrifuged at 17,000*g* for 5 min before HPLC analysis. For commercial probiotics, four different probiotic products were obtained from local pharmacy stores. The strains of these products are presented in Figure 2. The probiotics were homogenized with physiological saline to obtain solutions with the concentration of 10 billion cfu/mL.

5.3. HPLC Analysis. The HPLC system was a Waters 2695 instrument (Milford, MA, USA) with a quaternary pump, an automatic injector, a photodiode array detector (model 996), and Waters Empower software for peak identification and integration. Separations were carried out on a Phenomenex Prodigy ODS (2) column (250 × 3.2 mm, 5 μm). A binary gradient solvent system of acetonitrile (eluent A)−0.1% (v/v) phosphoric acid in water (eluent B) was used as follows: 12% A and 88% B (0−5 min), 20% A and 80% B (15 min), 45% A and 55% B (20−22 min), and 12% A and 88% B (25−30 min).

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The flow rate was 1.0 mL/min, and absorbance was detected at 272 nm. All tested solutions were filtered through Millex 0.2 μm nylon membrane syringe filters before use. The contents of the constituents were calculated using standard curves of references.

5.4. Cell Lines and Cultures. The human CRC cell lines HCT-116 and HT-29 (American Type Culture Collection, Manassas, VA, USA) were routinely cultured in McCoy's 5A medium supplemented with 10% FBS and 50 IU/mL penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were grown in a 25 mL flask and were routinely sub-cultured using 0.05% trypsin−EDTA solution every 3 days. In all experiments, cells were grown to 90−95% confluence and subjected to no more than 20 cell passages. Cells were maintained under the culture conditions described above for all experiments.

5.5. Cell Proliferation Analysis with the MTS Method. Ferulic acid and 4-vinylguaiacol were dissolved in dimethyl sulfoxide (DMSO) and were stored at 4 °C before use. Cells were seeded in 96-well plates (1×10^4 cells/well). After 24 h, the cells were treated with ferulic acid or 4-vinylguaiacol (0.25, 0.5, 1.0, and 1.5 mM) for 48 h. To observe the time- and concentration-dependency of the drugs, HCT-116 and HT-29 cells were treated under the same conditions as described above and incubated with ferulic acid or 4-vinylguaiacol (0.25, 0.5, 1.0, and 1.5 mM) for 24, 48, or 72 h. The final concentration of DMSO in the culture medium was 1%. Controls were exposed to culture medium containing 1% DMSO without test compounds. All experiments were conducted in triplicate and repeated three times. Following the indicated incubation period, cell proliferation was tested using an MTS assay according to the manufacturer's instructions. Briefly, the medium was replaced with 100 μL of fresh medium and 20 μL of MTS reagent (CellTiter 96 Aqueous Solution) in each well, and the plate was returned to be incubated for 2−4 h. Subsequently, 60 μL of medium from each well was transferred to an ELISA 96-well plate, and the absorbance was recorded at 490 nm. The results were expressed as percent of control (DMSO vehicle concentration is set as 100%).

5.6. Hoechst 33258 Staining Assay. Hoechst 33258 is commonly used to visualize the structure of the nucleus in fluorescence microscopy. Cells (1×10^5 cells/well in a 24-well plate) were treated with test compounds for 48 h. Then, cells were separated with trypsin and transferred into a 1.5 mL tube. After being centrifuged at 2000 rpm for 5 min, the supernatant was removed. The cells were stained with the PBS solution containing 0.01 mg/mL Hoechst 33258, 33 mg/mL formaldehyde, and 5 mg/mL NP-40 for 10 min in the dark. The cells were then observed using a fluorescence microscope with an excitation light at 365 nm.

5.7. Cell Cycle Analysis. Cells were seeded in 24-well plates. On the second day, the medium was replaced by fresh medium, and then the cells were treated with test compounds for 48 h before harvesting. These cells were fixed with 80% ethanol gently in a freezer for 2 h and were then treated with 0.25% Triton X-100. Cells were resuspended in PBS containing 0.1 mg/mL RNase and 40 μg/mL PI. After incubating in the dark for 20 min, cell cycle analysis was performed using an LSR II flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, ≥10,000 cells were counted.

5.8. Apoptotic Analysis. The apoptosis assay was performed by flow cytometry following a previously described procedure.[37] Briefly, cells were seeded in 24-well plates. After 24 h, the medium was replaced by a fresh medium, and test compounds were added. After treatment for 48 h, floating and adherent cells were collected. After centrifugation, the supernatant was removed, and cells were stained with annexin V-FITC and PI. The cells were immediately analyzed after staining using a LSR II flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, ≥20,000 cells were counted.

5.9. Caspase 3, 8, and 9 Analyses. HT-29 cells were seeded in 6-well plates. After 24 h, the medium was replaced by fresh medium and 4-vinylguaiacol was added. After treatment for 12 h, cell lysates were collected. Expression levels of caspases 3, 8, and 9 were determined by the colorimetric method according to the manufacturer's instructions. Briefly, cell lysates were diluted to a protein concentration of 0.5 mg/mL. Then, 5 μL of colorimetric tetrapeptide substrate (DEVD-pNA for caspase 3, IETD-pNA for caspase 8, and LEHDpNA for caspase 9) and cell lysate were added, and the plate was incubated at 37 °C for 24 h. Absorbance was recorded at 405 nm. The change in caspase activity was calculated as the absorbance of 4-vinylguaiacol-treated cells/absorbance of untreated controls.

5.10. Statistical Analysis. Data are presented as mean ± SE. One-way ANOVA was applied to determine the statistical significance of results. When necessary, Student's *t*-test was used to compare the two groups. The level of statistical significance was set as $P < 0.05$.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CRC, colorectal cancer; HPLC, high-performance liquid chromatography; MTS, modified trichrome stain; FA, ferulic acid; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PBS, phosphate-buffered solution; FITC, fluorescein isothiocyanate; PI, propidium iodide; LOD, limit of detection; LOQ, limit of quantitation; HFM, human fecal microflora

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