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Research Article

Ferulic acid inhibiting colon cancer cells at different Duke's stages

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Abstract: Ferulic acid is a widely distributed phenolic acid in plants and herbs, often conjugates with large molecules and enters the colon to exert anti-cancer effect. However, its suppression effect and mechanisms of action on colon cancer cells across various stages of Duke's classification is not clear. This study aims to investigate the effects of ferulic acid on the migration, cell cycle, apoptosis, and signaling pathways in colon cancer cells (SW-480, Caco-2, and HCT-116) at different Duke's stages. Results demonstrates that ferulic acid significantly inhibits the proliferation and migration of these cells, inducing cell cycle arrest at different phase, and ultimately promotes apoptosis in a dose-dependent manner. Specifically, ferulic acid activates the ATM/Chk2 and ATR/Chk1 pathways, down regulating their relative cell cycle regulatory proteins (CDK2 and Cyclin A2 complex, CDK4/6 and Cyclin D1/E1 complex), and thus leading to S-phase arrest in SW-480 and Caco-2 cells, and G1 phase arrest in HCT-116 cells, respectively. In addition, upregulated p53 and p21 proteins also contributing to the induction of apoptosis. This study is highly significant as they provide a deeper understanding of the molecular mechanisms by which ferulic acid exerts its anticancer effects at different Duke's stages, and propose novel dietary strategy for the prevention of colon cancer.

Keywords: ferulic acid; colon cancer cells; cell cycle regulatory proteins; apoptosis; Duke's stages

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1 Introduction

The incidence and death rate of colon cancer are within top 5 worldwide, as reported by IRIC (The International Agency for Research on Global Cancer Observatory) in 2022[1]. One of the most important reasons for the high incidence of colon cancer is reported to be the poor diet pattern[2]. Diet plays an important role in human health[3], especially colon cancer, attributing to the fact that small and large intestines are the predominant digestion and absorption site of foods. Bioactive compounds exist in foods could be released in the gut and thereby contributing to restoration of a disrupted intestinal environment.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a ubiquitous phenolic compound present in various plant food such as cereals, fruits, vegetables, coffee, etc.[4-6]. It often conjugates with large molecules and enters the colon[7] to exert health benefits. Ferulic acid exhibits potential anticancer effects by inhibiting the growth of cancer cells through suppression of proliferation, initiation of apoptosis and cell cycle arrest as well as modulating various signaling pathways[8]. However, the underlying mechanisms associated with colon cancer at different progressive stage is rarely compared. Duke's classification system is a well-established framework and utilized for categorizing colon cancers into four progressive stages: A, B, C, and D[9, 10]. Each stage reflects the extent of tumor invasion and the presence of metastasis[11]. Colon cancer cells at different progressive stages react to environmental stimuli differently[12].

Thus, this study aimed to elucidate the impact of ferulic acid on colon cancer cells across various stages of Duke's classification by examining its inhibitory effects on cells at three distinct levels. Specifically, SW-480 cells corresponded to Duke's Stage B, Caco-2 cells indicative to Duke's Stage C, and HCT-116 cells to Duke's Stage D, representing an increasing order of tumor progression. Furthermore, the potential signaling pathways and regulating proteins of the inhibition mechanisms were analyzed by Western blotting and reverse transcription-polymerase chain reaction (RT-PCR). Collectively, this study compares the reactions of colon cancer cells to ferulic acid treatment at various stages of progression, reveals the underlying mechanisms, and suggests innovative dietary pattern for prevention strategies of colon cancer.

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2 Materials and methods

2.1 Materials and chemicals

Three human colon cancer cell lines (SW-480, Caco-2, and HCT-116) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Ferulic acid standard was obtained from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-GAPDH, anti-Cyclin A2, anti-Cyclin D1, anti-Cyclin E1 and anti-p53 were provided by Bioss (Beijing, China). The primary antibodies against ataxia-telangiectasia mutated (ATM), ATM and Rad3-related (ATR), checkpoint kinase 1 (Chk1), Chk2, cyclin-dependent kinase 2 (CDK2), CDK4, CDK6, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Cyclin A2, Cyclin D1, Cyclin E1, p53, p21, p16, retinoblastoma 1 (Rb1), E2F transcription factor 1 (E2F1) were purchased from Wuhan Tianyi Huayu Gene Technology Co., Ltd. (Wuhan, China). RIPA lysate, phenylmethylsulfonyl fluoride (PMSF), BCA assay, CCK-8 kit, total RNA isolation reagent, reverse transcription kit, and universal SYBR qPCR master mix were obtained from Biosharp (Hefei, China). All chemicals and reagents were of analytical grade unless otherwise described.

2.2 Cell culture

SW-480, HCT-116, and Caco-2 cell lines were cultured with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2 humidified atmosphere. Cells were sub-cultured or collected at 80% confluence. The exponential growth phase cells between passages 5 and 30 were used for the following experiments.

2.3 Effect of ferulic acid on cancer cell proliferation

2.3.1 *Cell viability*

Cell viabilities of SW-480, HCT-116, and Caco-2 cell lines were assessed by a CCK-8 assay kit (Biosharp, China) according to our previous work[13]. An aliquot of 100 μL cells was seeded in a 96-well plate at a density of 4×10^4–5×10^4 cells/mL and treated with ferulic acid at 0–400 μg/mL for 24, 48, and 72 h respectively. The culture medium was removed at each time point, replaced with a serum-free medium containing 10% (*V*/*V*) CCK-8 solution, and incubated at 37 °C for 2 h before measuring. Absorbance was read at 450 nm using a microplate reader (Per Elmer EnSpire, MA, USA). Oxaliplatin (OXA), a commercial anti-colon cancer drug, was used as a positive control. Cell viability was calculated by the following equation:

$$\text{Cell viability}\,(\%) = \frac{A_{450\text{ nm Sample}} - A_{450\text{ nm Blank}}}{A_{450\text{ nm Control}} - A_{450\text{ nm Blank}}} \times 100$$

2.3.2 *Colony formation assay*

Colony formation of SW-480, HCT-116, and Caco-2 cell lines was tested according to previously reported the method[14]. Cells treated with ferulic acid at 0, 200, 400 μmol/L were collected and re-seeded into 6-well plates at a density of 1,000 cells/well. The medium was changed every 3 days for 14 days until visible colonies formed. Afterwards, the colonies in each well were fixed with 1 mL 4% methanol, stained with crystal violet (0.1%) for 30 min, and counted by ImageJ software (V1.8.0.112).

2.4 Effect of ferulic acid on cancer cell migration

2.4.1 *Wound healing assay*

Wound healing assay on cells was performed as previously described by Zhang and co-workers[15] with some modifications. Cells (5×10^5 cells/well) were seeded into a 6-well plate and cultured until confluency. The formed cell monolayers were then scratch using a 10 μL sterile pipette tip and washed with PBS to remove the cellular debris. The obtained wounded cell monolayers were then treated with ferulic acid (0, 200, 400 μmol/L) for 24 h. Finally, the cells were washed with PBS and fixed with paraformaldehyde before being photographed under a biological microscope (BH-RFL, Sunny Optical Technology (Group) Co., Ltd., Ningbo, China) with a high-resolution digital camera. Percentage of migration was calculated by counting the number of cells that migrated into scratched areas compared with cells that stayed in the peripheral areas through ImageJ software (version 1.80) analysis system.

2.4.2 *Transwell migration assay*

Cell migration assay was performed according to Cao *et al.*[16] with some modifications using a Labsecelect 24-well chamber of pore size 8 μm. Ferulic acid (0, 200, 400 μg/mL for 72 h) treated cells (2×10^5 cells/well) in FBS free DMEM medium were added into the upper chamber. DMEM with 30% FBS was in the lower chamber for inducing cell migration. After incubation for 24 h, cells in the upper chamber were removed with cotton swabs, and the cells that migrated to the lower surface of chamber membrane was fixed in methanol and counted under a microscope at a magnification of 20× in five random fields.

2.5 The suppression mechanism

2.5.1 *Cell cycle and apoptosis analysis*

Cell cycle and apoptosis analysis were conducted as previously reported[17]. Caco-2 cells were seeded at 1×10^6 cells/well in a 6-well plate and incubated at 37 °C in 5% CO_2 before being treated with ferulic acid at 400 μg/mL for 72 h.

For cell cycle analysis, treated cells were detached and fixed in 70% ice cold ethanol at 4 °C, and then resuspended in 10 μL of propidium iodide (PI, 250 μg/mL) and 10 μL of RNase solution at 37 °C for 30 min before analysis by a flow cytometer (cytoFLEX, Beckman Coulter, Inc., Kraemer Boulevard Brea, CA, USA) with excitation at 488 nm and emission at 617 nm.

For apoptosis assay, treated cells were collected and fixed in 1% paraformaldehyde and 70% ice cold ethanol at 4 °C, and then 5 μL of AnnexinV-FITC and 5 μL of PI were added. After 10 min of incubation, the apoptosis of treated cells was analyzed by a flow cytometer with excitation at 488 nm and emission at 350 nm.

2.5.2 *Western blot analysis*

Western blotting is a technique for detecting protein developed based on protein electrophoresis separation and antigen-antibody detection[18]. Western blot analysis was conducted as previously reported, with some modifications[19,20]. Cells were treated with 0, 200, or 400 μg/mL ferulic acid 1 day after seeding and collected after 72 h of treatment, then lysed with RIPA containing 1 mmol/L PMSF (Biosharp, Hefei, China). Protein concentrations were assessed by BCA protein assay kit (Biosharp, Hefei, China).

After counting in a hemocytometer, the cells were pelleted at 750 *g* for 10 min at 4 °C and stored at −80 °C. The cells were diluted in sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% *β*-mercaptoethanol), incubated at 100 °C for 10 min, and were then put on ice. Cells were loaded 100,000 cells in 10 μL in 10-well precast 10% SDS polyacrylamide gels (for detection of Cyclin D1, Cyclin E1, Cyclin A2, and P53 proteins), and the proteins were separated electrophoretically and then transferred to a nitrocellulose membrane by electroblotting. The membranes were blocked with 5% dry milk and 0.05% Tween-20 in PBS for 1 h followed by incubation for 2 h with the primary antibody at 20 °C. All membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, and the enhanced chemiluminescence (ECL) detection reagent was used according to the manufacturers' protocol. The ChemiDoc XRS system (Bio-Rad, Inc., Hercules, CA, USA) was used for imaging.

2.5.3 *RT-qPCR analysis*

The total RNA was isolated from experimental cells using TRIzol reagent (Biosharp, China) according to the manufacturer's instructions. The extracted total RNA was converted into complementary DNA (cDNA) in order to maintain the purity of RNA. The cDNA was synthesized from 1 ng of the total RNA using reverse transcriptase RT-qPCR enzyme kit (Biosharp, China). Primers were designed using the public resource for PCR primers (Primerbank), as shown in Table 1. The real-time PCR was performed on the CFX96 real-time system (Bio-Rad, Inc., Hercules, CA), with SYBR Green PCR master mix (Biosharp, China) according to the manufacturer's guidelines. All raw data of gene expressions were normalized to the expression level of the housekeeping gene, the *GAPDH*.

2.6 Statistical analysis

The experimental results were expressed as average ± standard deviation (SD). Each experiment was repeated 3 times independently. Data were analyzed by one-way ANOVA and Duncan's multiple range test at a 5% significance level ($P < 0.05$) using SPSS statistics program (SPSS Inc., Chicago, IL, USA).

3 Results and discussion

3.1 Ferulic acid reduces cell viability and impairs colony formation capacity

The cell viability of three colon cancer cell lines (SW-480, Caco-2, and HCT-116) treated by ferulic acid at 0–400 μg/mL up to 72 h was measured by a CCK-8 kit. OXA, a commercial anti-cancer drug, was used as the positive control. In general, treatment of ferulic acid at various concentrations showed a dose-dependent reduction in the cell viability at both 48 and 72 h (Fig. 1). Although the cell viability exceeded 100% over time, ferulic acid predominantly impedes cell proliferation, leading to a deceleration in cell growth rather than the immediate induction of cell death. This observation is consistent with the findings of Ashraf *et al.*[21] and may be attributed to the influence of the cell cycle on cell fate decisions, which can result in outcomes such as senescence or delayed apoptosis rather than immediate cell death.

The calculated Pearson correlation coefficient (*r*), as shown in Table 2, exhibited a strong negative correlation between treating dosage of ferulic acid and cell viability at 72 h. Specifically, cell viability in HCT-116 cells was significantly reduced at a ferulic acid concentration of 25 μg/mL following 48 and 72 h of exposure. In contrast, no significant differences in cell viability were observed in SW-480 and Caco-2 cells at concentrations below 100 μg/mL. Meanwhile, the calculated half maximal inhibitory concentration (IC_{50}) values at 72 h (Table 2) showed a lowest value of (165.46 ± 5.87) μg/mL for HCT-116 cells. These findings indicate that ferulic acid demonstrates a superior efficacy in inhibiting HCT-116 cell proliferation, aligning with the study by El-Gogary *et al.*[22], which employed a ferulic acid nano-capsule for the treatment of various colon cancer cell lines. In addition, OXA also showed a lowest IC_{50} value for HCT-116 cell line, which could be reasonably attributed to the varying sensitivities or response of different colon cancer cell lines to a specific drug[23].

To evaluate the efficacy of ferulic acid in inhibiting the proliferation of colon cancer cells, colony formation assay was conducted on SW-480, Caco-2, and HCT-116 cells treated with 0, 200, and 400 μg/mL ferulic acid. The results indicated a significant reduction in colony formation across all three cell lines with

Table 1 Primers of genes used for RT-qPCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
GAPDH	TGTGGGCATCAATGGATTTGG	ACACCATGTATTCCGGGTCAAT
ATM	TTGATCTTGTGCCTTGGCTAC	TATGGTGTACGTTCCCCATGT
ATR	TCCCTTGAATACAGTGGCCTA	TCCTTGAAAGTACGGCAGTTC
Chk1	ATATGAAGCGTGCCGTAGACT	TGCCTATGTGTGGCTCTCTATTCTG
Chk2	TTATCTGCCTTAGTGGGTATCCA	CTGTCGTAAAACGTGCCTTTG
p53	GAGGTTGGCTCTGACTGTACC	TCCGTCCCAGTAGATTACCAC
p21	CGATGGAACTTCGACTTTGTCA	GCACAAGGGTACAAGACAGTG
p16	GATCCAGGTGGGTAGAAGGTC	GATCCAGGTGGGTAGAAGGTC
CDK2	GTACCTCCCCTGGATGAAGAT	CGAAATCCGCTTGTTAGGGTC
CDK4	ATGGCTACCTCTCGATATGAGC	CATTGGGGACTCTCACACTCT
CDK6	CCAGATGGCTCTAACCTCAGT	AACTTCCACACGAAAAAGAGGCTT
Cyclin A2	GGATGGTAGTTTTGAGTCACCAC	CACGAGGATAGCTCTCATACTGT
Cyclin D1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACATTTGAA
Cyclin E1	GCCAGCCTTGGGACAATAATG	CTTGCACGTTGAGTTTGGGT
Rb1	CTCTCGTCAGGCTTGAGTTTG	GACATCTCATCTAGGTCAACTGC
E2F1	CATCCCAGGAGGTCACTTCTG	GACAACAGCGGTTCTTGCTC

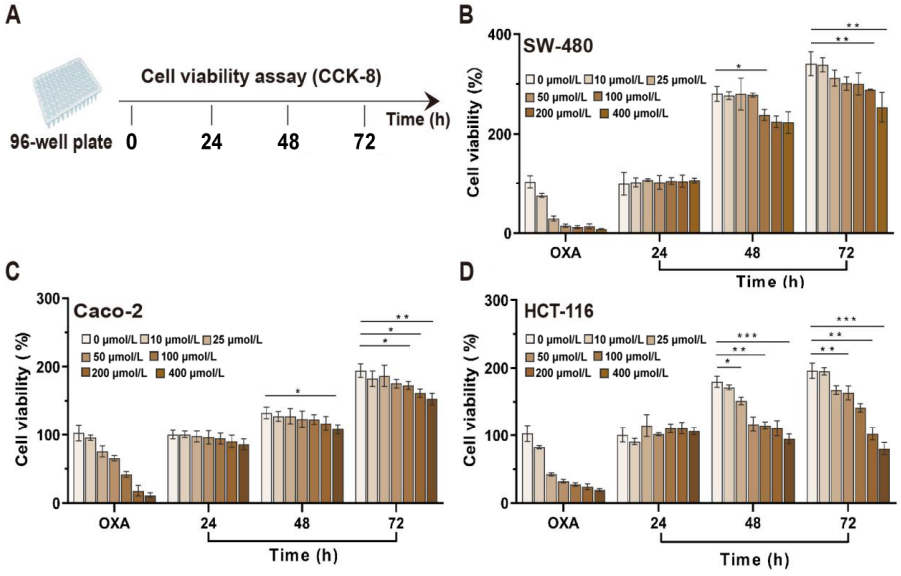

Figure 1 Cell viability of SW-480, Caco-2, and HCT-116 cells treated by ferulic acid at 0–400 μg/mL. OXA, a commercial anti-cancer drug, was used as a positive control. Significance level was denoted as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The same below.

Table 2 IC_{50} values and Pearson correlation coefficient (r) between treating dosage of ferulic acid and cell viability at 72 h of treatment

Cell lines	IC_{50} (μg/mL)		Pearson correlation coefficient (r)
	Treated by ferulic acid	Treated by OXA	
SW-480	176.41 ± 16.77	34.93 ± 1.17	−0.923 (P = 0.003)
Caco-2	179.19 ± 25.71	52.26 ± 6.06	−0.922 (P = 0.003)
HCT-116	165.46 ± 5.87	21.25 ± 2.04	−0.943 (P = 0.001)

increasing doses of ferulic acid (Fig. 2), suggesting that ferulic acid effectively suppresses the growth of different colon cancer cells. Similar results were reported in another human colon cancer cell line HT-29[24], as well as some other cancer cell models such as anti-esophageal squamous cell carcinoma (ESCC) cells[16], pancreatic cancer cells[25], and medullary thyroid carcer cells[26], and lung cancer cells[27].

3.2 Ferulic acid inhibits the cell migration activity of colon cancer cells

To examine the impact of ferulic acid on the migratory capacity of colon cancer cells, wound healing and Transwell migration assays were performed on those cells cultured for 72 h. The wound healing assay was conducted according to Kumar *et al.*[28]. The results, as depicted in Fig. 3A, demonstrate that the wound healing areas of SW-480, Caco-2, and HCT-116 cells were reduced by (10.69 ± 1.28)%, (38.29 ± 4.46)%, and (14.37 ± 0.77)%, respectively, following treatment with 400 μg/mL ferulic acid in comparison to the vehicle control (0 μg/mL of ferulic acid in the media) ($P < 0.05$). Similar study conducted by Fahrioğlu *et al.*[25] have corroborated that ferulic acid inhibit cancer cell migration using human pancreatic cancer cells (MIA PaCa-2). These findings suggest that ferulic acid possesses the capability to

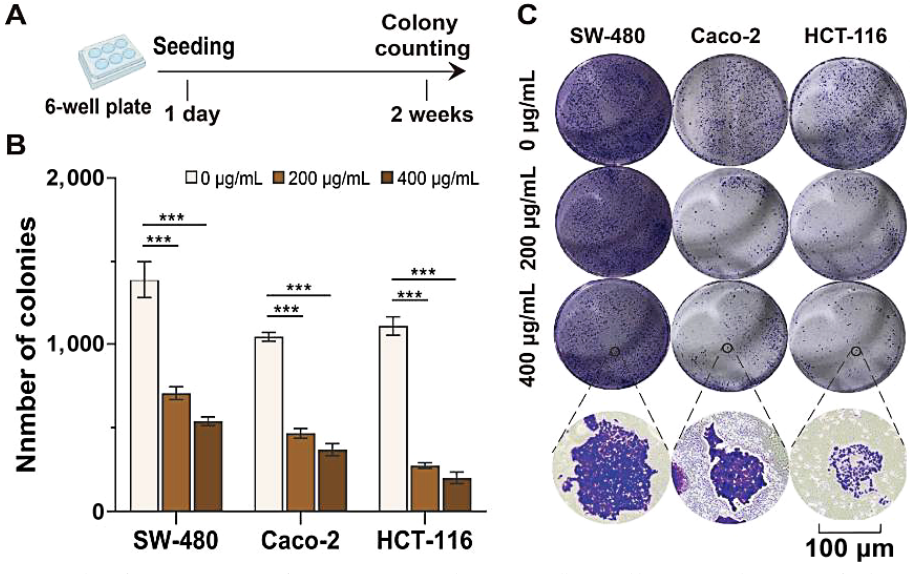

Figure 2 Colony formation capacity of SW-480, Caco-2, and HCT-116 cells treated by 0, 200, and 400 μg/mL ferulic acid.

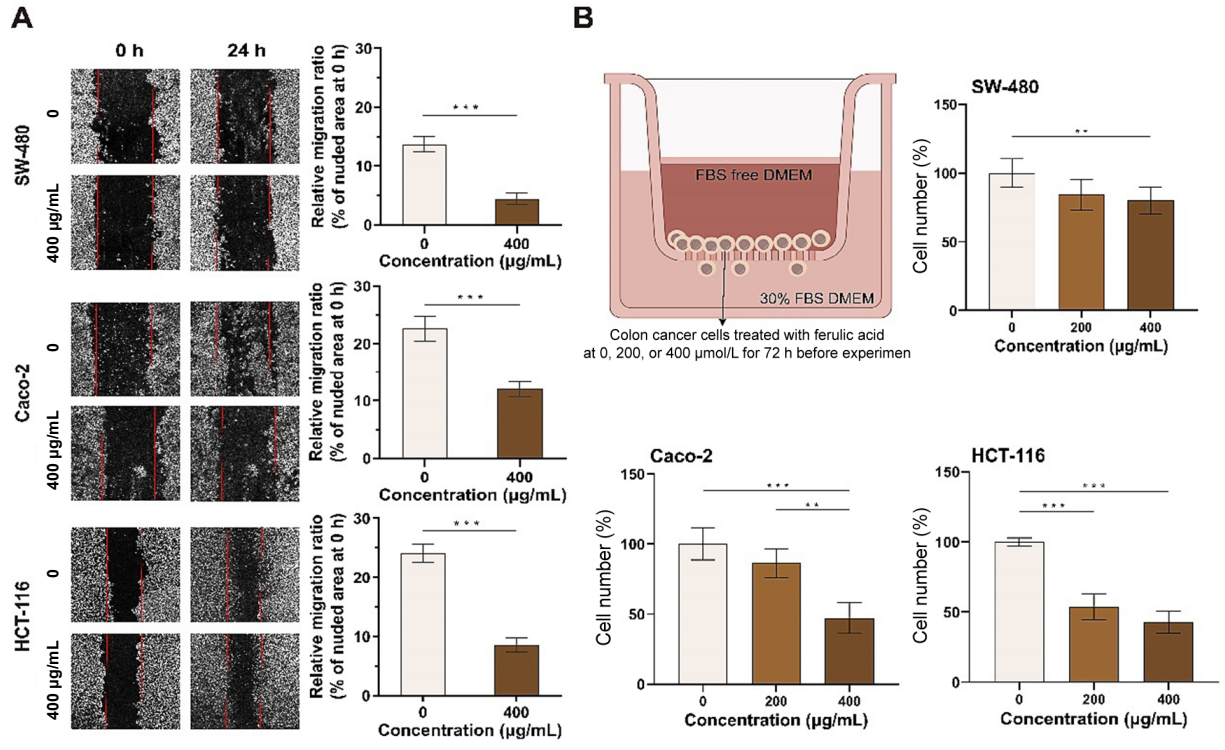

Figure 3 Effect of ferulic acid (400 μg/mL) on the migration capability of the cells.

suppress cancer cell metastasis *in vitro*.

To further validate the impact of ferulic acid on cellular migration, a transwell migration assay was introduced. According to Fig. 3B, ferulic acid effectively reduced the number of migrated cells in the Transwell and significantly suppressed the migration of the three cell lines in a dose-dependent manner over a 72-h period. This finding is in line with esophageal squamous carcinoma[16] and human prostate cancer cells[29] that treated with ferulic acid. Cell proliferation and migration are implicated in a range of pathological processes, from atherosclerosis to cancer[30]. Ferulic acid has been demonstrated to influence various functions of cancer cells *in vitro*, including cell proliferation, viability, migration, and sensitivity to radiotherapy or chemotherapy[29, 31-34]. Consequently, the underlying inhibitory mechanism warrant further exploration, and ferulic acid holds significant potential as a therapeutic agent for colon cancer.

3.3 Ferulic acid differently impact the cell cycle progression and apoptosis in SW-480, Caco-2, and HCT-116 cells

As illustrated in Fig. 4A, a substantial proportion of SW-480 and Caco-2 cancer cells were arrested in the S phase in a dose-dependent manner following ferulic acid treatment, whereas HCT-116 cells arrested in the G1 phase. These findings indicated the critical association between cell cycle regulation and tumorigenesis following the treatment of ferulic acid.

In details, after 72 h of treatment, the proportion of Caco-2 cells at S phase significantly rising from approximately (11.30 ± 0.68)% in the vehicle control to (28.48 ± 4.08)% and (37.11 ± 1.75)% for the 200 and 400 μg/mL treatment groups, respectively ($P < 0.05$). This finding is in agreement with Birgit *et al.*[33] that ferulic acid induces a delay in the S phase of Caco-2 cells, attributing to the up-regulation of the corresponding checkpoint protein SMC1L1. Similar phenomenon was found in SW-480 cells. Different from these two cell lines, the proportion of HCT-116 cells was elevated at G1 phase, which is in line with Wang *et al.*[35] using osteosarcoma cells and possibly because of the down-regulation of Cyclin D1. These findings collectively underscore the significance of checkpoint proteins and their regulatory roles in cell cycle control, particularly in response to external stimuli such as ferulic acid.

In addition, the interaction between ferulic acid and the cellular mechanisms governing cell cycle progression, such as the regulation of cyclins and cyclin-dependent kinases, may vary among different colon cancer cell lines, attributing to their distinct genetic expression profiles and signaling pathways[36].

For instance, the activation of the ATR-Chk1 pathway in SW-480 and Caco-2 cells may lead to cell cycle arrest in the S phase. Meanwhile, the activation of the ATM-Chk2 pathway, along with the downregulation of Cyclin D1 and Cyclin E1 proteins in HCT-116 cells, may result in cell cycle arrest in the G1 phase[37, 38].

To examine the impact of ferulic acid on apoptosis in colon cancer cells, the Annexin V/PI assay was employed to quantify the proportion of cells undergoing apoptotic processes. Following treatment with ferulic acid, dose-dependent increase of the apoptotic cells was observed (Fig. 4B) in the three cell lines. This result implied that ferulic acid can effectively induce apoptosis in colon cancer cells. Specifically, Caco-2 and SW-480 cells exhibited a greater tendency for undergoing apoptosis in comparison to HCT-116 cells, as evidence by higher apoptotic rates. Growing evidence suggests that the pro-apoptotic effect of ferulic acid on cancer cell lines is associated with the p53-p21 signaling pathway[39].

Research has shown that ferulic acid induces apoptosis and caused cell cycle arrest at the G1 phase by upregulating the expression of p53 and p21 proteins, accompanied by a concomitant decrease in the levels of Cyclin D1 and Cyclin E in HeLa and Caski cervical carcinoma cell lines[38]. Additionally, Reddy *et al.*[40] revealed that ferulic acid enhances the expression of proapoptotic proteins p53 and Bax, arresting the cell cycle and

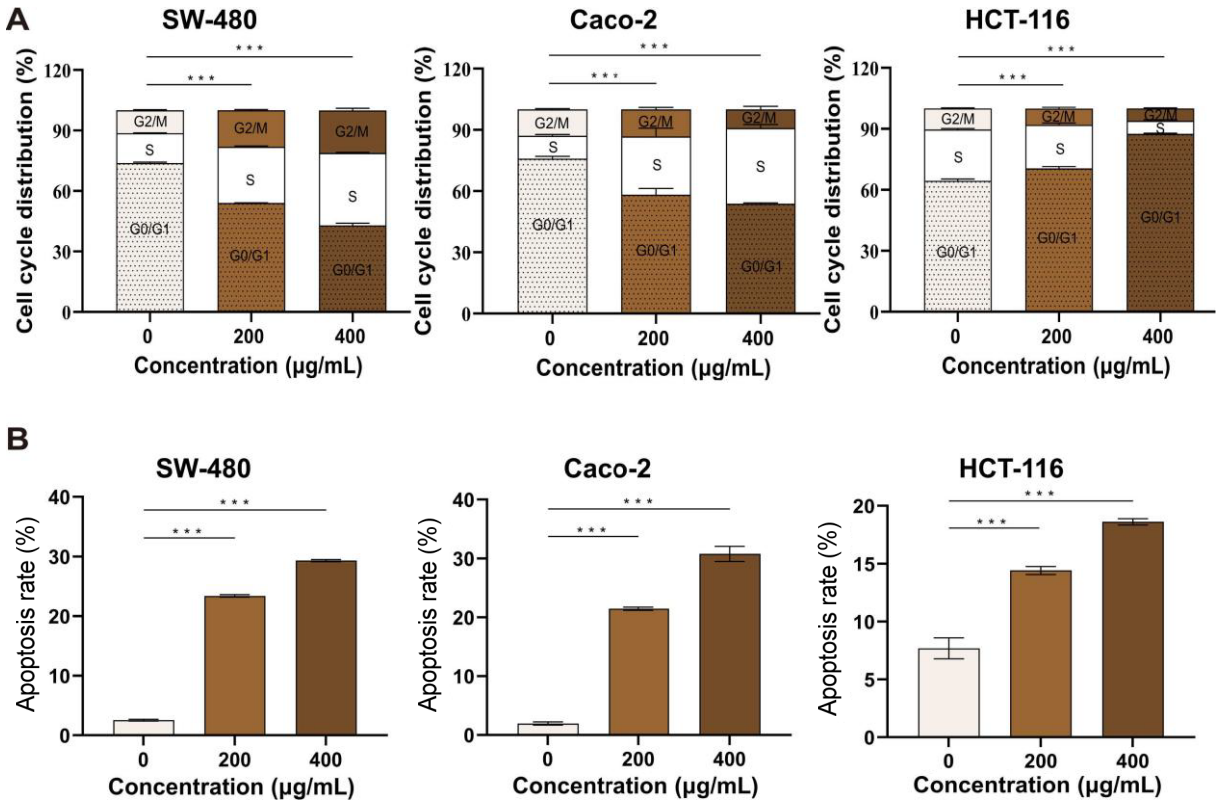

Figure 4 Effect of ferulic acid (0, 200, and 400 μg/mL) on cell cycle and apoptosis. (A) Cell cycle distribution of SW-480, Caco-2, and HCT-116 cells after treatment with ferulic acid. (B) Percentage of cells underwent apoptosis after treatment with ferulic acid.

overcoming apoptosis resistance in human lung cancer cell lines NCI-H460 and NSCLC. However, investigations into its effects on colon cancer cells at different Duke's stages remain limited. Therefore, further investigation is warranted into the molecular mechanisms by which ferulic acid influences signaling pathways that regulate the proliferation of human colon cancer cells.

3.4 Ferulic acid impact regulating proteins of cell cycle and apoptosis

To investigate the impact of ferulic acid on cell cycle and apoptosis regulation proteins, the expression levels of Cyclin A2, Cyclin D1, Cyclin E1, and p53 were assessed in SW-480, Caco-2, and HCT-116 cells following 72 h of treatment with ferulic acid, utilizing western blot analysis. Results showed in Fig. 5 revealed that ferulic acid induces a dose-dependent reduction in the expression of key proteins associated with cell cycle arrest, specifically Cyclin A2, thereby corroborating the observed S phase cell cycle arrest in SW-480 and Caco-2 cells discussed in Section 3.3. Cyclin A has been reported to play a role in regulating the initiation of DNA synthesis during the S phase[41-43]. While in HCT-116 cells, a dose-dependent decrease in Cyclin D1 and

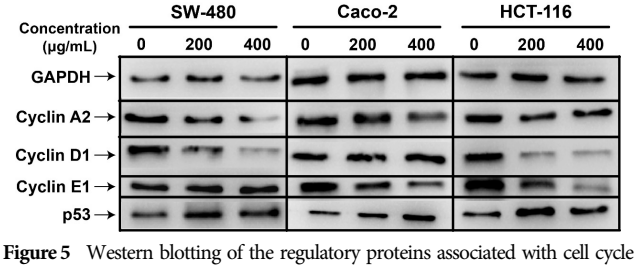

Figure 5 Western blotting of the regulatory proteins associated with cell cycle and apoptosis in SW-480, Caco-2, and HCT-116 cells after ferulic acid treatment.

Cyclin E1 protein was observed. This finding explained the phenomenon that HCT-116 cells were arrested at G1 phase as depicted in Fig. 4, since Cyclin D is responsible for the progression of cell cycle through the G1 phase and Cyclin E is responsible for triggering the transition from late G1 into S phase[44, 45]. Furthermore, ferulic acid treatment resulted in the upregulation of p53 protein expression across the three cell lines, thereby facilitating both cell cycle disruption and the induction of apoptosis. The transcription factor p53 is pivotal in preserving genomic integrity within cells. In response to genomic damage, p53 arrests the cell cycle to enable DNA repair mechanisms and activates the expression of target genes such as p21 and BAX, which are involved in the regulation of the cell cycle and apoptosis, respectively[46].

In summary, the analysis of key regulating proteins in SW-480 and Caco-2 cells demonstrated that ferulic acid significantly reduce Cyclin A2 expression, corroborating our findings of S phase arrest following ferulic acid treatment. While the downregulating of Cyclin D1 and Cyclin E1 contributed to the G1 phase arresting in HCT-116 cells. Additionally, the increased expression of p53 promoted apoptosis in the three cell lines further leads to apoptosis and confirmed the regulatory impact of ferulic acid to colon cancer cells.

3.5 RT-qPCR analysis

RT-qPCR was employed to evaluate the expression levels of relevant genes to elucidate the signaling pathway potentially influenced by ferulic acid. The panels utilized in this study comprise genes including *ATM*, *ATR*, *Chk1*, *Chk2*, *CDK2*, *CDK4*, *CDK6*, *GAPDH*, *Cyclin A2*, *Cyclin D1*, *Cyclin E1*, *p53*, *p21*, *p16*, *Rb1*, and *E2F1*. Results were showed in Fig. 6. Following a 72-h treatment with ferulic acid, the expression levels of *ATR* and *Chk1*

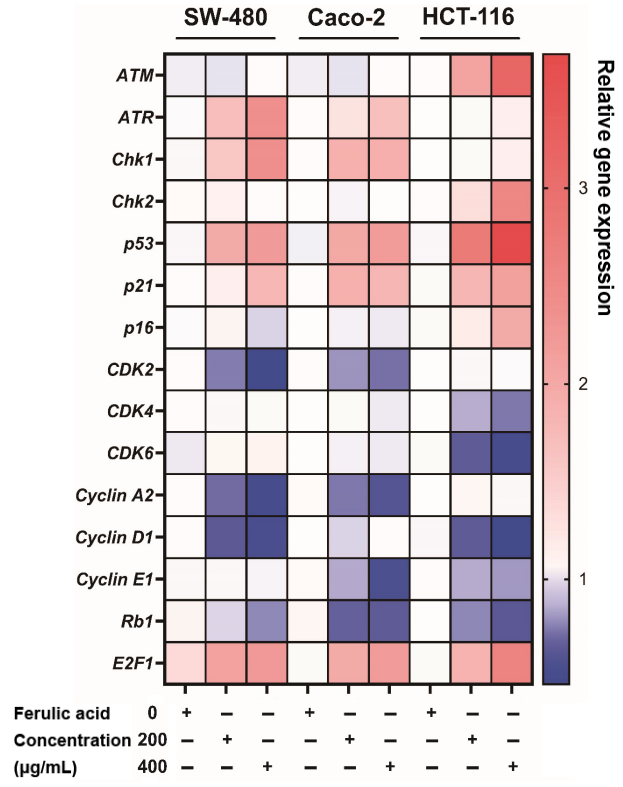

Figure 6 Impact of ferulic acid at 200 or 400 μg/mL on the corresponding gene expression of the regulatory proteins of cell cycle and apoptosis analyzed by RT-qPCR.

were observed to be up-regulated, whereas *CDK2* was down-regulated in SW480 and Caco-2 cells. The activation of *ATR-Chk1* in SW-480 and Caco-2 cells leads to the phosphorylation and subsequent deactivation of *CDC25*. This process, as illustrated in Fig. 6, leads to the downregulation of *CDK2* expression and a subsequent reduction in the *Cyclin A2-CDK2* complex, ultimately resulting in cell cycle arrest at the S or G2/M phase[47,48]. As in HCT-116 cells, ferulic acid treatment led to a reduction in the expression levels of *CDK4* and *CDK6*, facilitating the ubiquitination-mediated degradation of *Cyclin D* and causing cell cycle arrest at the G1 phase[49]. Concurrently, the activation of *ATM* may further stimulate *Chk2*. Upon activation, *Chk2* facilitates the ubiquitin-mediated degradation of the phosphatase *CDC25A*, resulting in the inactivation of *CDK2-Cyclin E* complexes, and consequently contributes to cell cycle arrest[50].

Furthermore, the expression levels of p53 and p21 were up-regulated in the three cell lines, which might attribute to the up-regulated *ATM/ATR* that results in phosphorylation and subsequent accumulation of *p53*, and in turn induces the synthesis of the CDK inhibitor *p21*. This inhibitor interacts with *CDK2/Cyclin E* and *CDK2/Cyclin A* complexes, thereby inhibiting their activities and resulting in arrest of the cell and apoptosis[37,51-54]. In addition, p53 has been reported inhibit colon cancer cells by activating *Bax* and down-regulating *Bcl2* that reported to be associated with apoptosis of cancer cells[55].

3.6 Proposed mechanism of ferulic acid in suppressing colon cancer cells

A proposed mechanism by which ferulic acid inhibits different colon cancer cells is illustrated in Fig. 7. Ferulic acid is commonly found in numerous plant foods, often exiting in a bound form within the food matrix. These food matrices facilitate the delivery of ferulic acid into the colon, where it is released to exert anti-

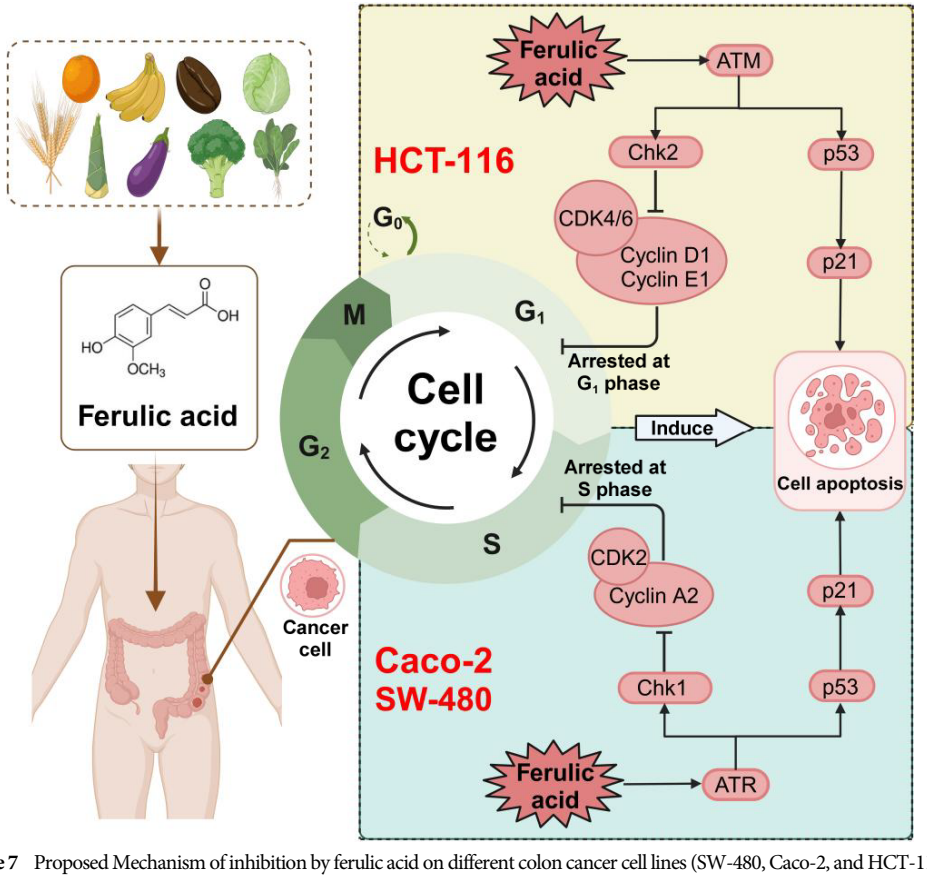

Figure 7 Proposed Mechanism of inhibition by ferulic acid on different colon cancer cell lines (SW-480, Caco-2, and HCT-116).

cancer effect. Exposure of colon cancer cells to ferulic acid results in the different suppression pathways within different cell types. SW-480 and Caco-2 cells, corresponding to Duke's Stage B and C, respectively, can be arrested in the G1 phase via the ATM/Chk2 pathway, which downregulates CDK2 and cyclin A2 complex. While HCT-116 cells, representing for Duke's Stage D, can be arrested at S phase through the ATR/Chk1 pathway, which leads to downregulation of cell cycle regulatory proteins CDK4/6 and cyclin D1/E1 complex, and further inducing apoptosis. In addition, the upregulated p53 and p21 also contributed to the anti-cancer activity of ferulic acid by induction of apoptosis in cancer cells.

4 Conclusion

Our data demonstrate that ferulic acid exerts inhibitory effects on cell proliferation, diminishes migratory capacity, and induces cell cycle arrest and apoptosis in human colon cancer cell lines Caco-2, HCT-116, and SW-480. The underlying mechanisms are associated with different pathways in the cells at different Duke's stages. In details, SW-480 and Caco-2 cells suppressed by ferulic acid through the ATR/Chk1 pathway, whereas HCT-116 cells via the ATM/Chk2 pathway. Upregulated p53 and p21 proteins also contributing to the suppression effects of ferulic acid. Taken together, these results suggest that the incorporating ferulic acid-rich foods may serve as a potential strategy for colon cancer prevention, and this approach offers an innovative measure for the development of functional foods that align with the principles of food and medicine homology.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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References

[1] Nierengarten, M. B. Global cancer statistics 2022. *Cancer*, **2024**, 130: 2568. https://doi.org/10.1002/cncr.35444

[2] Arnold, M., Sierra, M. S., Laversanne, M., et al. Global patterns and trends in colorectal cancer incidence and mortality. *Gut*, **2017**, 66: 683–691. https://doi.org/10.1136/gutjnl-2015-310912

[3] Stanaway, J. D., Afshin, A., Ashbaugh, C., et al. Health effects associated with vegetable consumption: a Burden of Proof study. *Nature Medicine*, **2022**, 28: 2066–2074. https://doi.org/10.1038/s41591-022-01970-5

[4] Graf, E. Antioxidant potential of ferulic acid. *Free Radical Biology and Medicine*, **1992**, 13: 435–448. https://doi.org/10.1016/0891-5849(92)90184-I

[5] Clifford, M. N. Chlorogenic acids and other cinnamates–nature, occurrence, dietary burden, absorption and metabolism. *Journal of the Science of Food and Agriculture*, **2000**, 80: 1033–1043. https://doi.org/10.1002/(SICI)1097-0010(19990301)79:3

[6] Mattila, P., Hellström, J. Phenolic acids in potatoes, vegetables, and some of their products. *Journal of Food Composition and Analysis*, **2006**, 20: 152–160. https://doi.org/10.1016/j.jfca.2006.05.007

[7] Zhang, B., Zhang, Y., Li, H., et al. A review on insoluble-bound phenolics in plant-based food matrix and their contribution to human health with future perspectives. *Trends in Food Science & Technology*, **2020**, 105: 347–362. https://doi.org/10.1016/j.tifs.2020.09.029

[8] Ashutosh, G., Kumar, S. A., Mariam, L., et al. Ferulic acid-mediated modulation of apoptotic signaling pathways in cancer. *Advances in Protein Chemistry and Structural Biology*, **2021**, 125: 215–257. https://doi.org/10.1016/BS.APCSB.2020.12.005

[9] Payne, J. E. International colorectal carcinoma staging and grading. *Diseases of the Colon & Rectum*, **1989**, 32: 282–285. https://doi.org/10.1007/BF02553480

[10] Ahmed, D., Eide, P.W., Eilertsen, I. A., et al. Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis*, **2013**, 2: e71. https://doi.org/10.1038/oncsis.2013.35

[11] Kitamura, K., Shida, D., Sekine, S., et al. Comparison of model fit and discriminatory ability of the 8th edition of the tumor-node-metastasis classification and the 9th edition of the Japanese classification to identify stage III colorectal cancer. *International Journal of Clinical Oncology*, **2021**, 26: 1671–1678. https://doi.org/10.1007/s10147-021-01955-3

[12] Berk, Ş., Janssen, J. A., van Koetsveld, P. M., et al. Modifying effects of glucose and insulin/insulin-like growth factors on colon cancer cells. *Frontiers in Oncology*, **2021**, 11: 645732. https://doi.org/10.3389/FONC.2021.645732

[13] Chen, X., Tang, W., Li, X., et al. Effect of extrusion on phenolics from Jizi439 black wheat bran: the profile, structure, and bioactivities. *LWT*, **2023**, 177: 114369. https://doi.org/10.1016/J.LWT.2022.114369

[14] Chen, X., Tian, J., Su, G. H., et al. Blocking IL-6/GP130 signaling inhibits cell viability/proliferation, glycolysis, and colony forming activity in human pancreatic cancer cells. *Current Cancer Drug Targets*, **2019**, 19: 417–427. https://doi.org/10.2174/1568009618666180430123939

[15] Zhang, X., Lin, D. A. N., Jiang, R., et al. Ferulic acid exerts antitumor activity and inhibits metastasis in breast cancer cells by regulating epithelial to mesenchymal transition. *Oncology Reports*, **2016**, 36: 271–278. https://doi.org/10.3892/or.2016.4804

[16] Cao, Y., Zhang, H., Tang, J., et al. Ferulic acid mitigates growth and invasion of esophageal squamous cell carcinoma through inducing ferroptotic cell death. *Disease Markers*, **2022**, 1: 4607966. https://doi.org/10.1155/2022/4607966

[17] Mazewski, C., Liang, K., Mejia, E. G. D. Comparison of the effect of chemical composition of anthocyanin-rich plant extracts on colon cancer cell proliferation and their potential mechanism of action using *in vitro, in silico*, and biochemical assays. *Food Chemistry*, **2018**, 242: 378–388. https://doi.org/10.1016/j.foodchem.2017.09.086

[18] Wang, F., Yang, G., Song, H., et al. The guideline for western blotting assay. *Food Frontiers*, **2022**, 3: 347–349. https://doi.org/10.1002/fft2.151

[19] Chen, X., Guo, Y., Jia, G., et al. Ferulic acid regulates muscle fiber type formation through the Sirt1/AMPK signaling pathway. *Food & Function*, **2019**, 10: 259–265. https://doi.org/10.1039/c8fo01902a

[20] Chen, X., Luo, Y., Huang, Z., et al. Akirin2 regulates proliferation and differentiation of porcine skeletal muscle satellite cells via ERK1/2 and NFATc1 signaling pathways. *Scientific Reports*, **2017**, 7: 45156. https://doi.org/10.1038/srep45156

[21] Ashraf, H. M., Moser, J., Spencer, S. L. Senescence evasion in chemotherapy: a sweet spot for p21. *Cell*, **2019**, 178: 267–269. https://doi.org/10.1016/j.cell.2019.06.025

[22] El-Gogary, R. I., Nasr, M., Rahsed, L. A., et al. Ferulic acid nanocapsules as a promising treatment modality for colorectal cancer: preparation and *in vitro*/*in vivo* appraisal. *Life Sciences*, **2022**, 298: 120500. https://doi.org/10.1016/J.LFS.2022.120500

[23] Naomi, I., Takeshi, T., Yuri, T. K., et al. The benzylisoquinoline alkaloids, berberine and coptisine, act against camptothecin-resistant topoisomerase I mutants. *Scientific Reports*, **2021**, 11: 7718. https://

doi.org/10.1038/S41598-021-87344-2
[24] Kumar, C. S., Thangam, R., Mary, S. A., et al. Targeted delivery and apoptosis induction of trans-resveratrol-ferulic acid loaded chitosan coated folic acid conjugate solid lipid nanoparticles in colon cancer cells. *Carbohydrate Polymers*, **2020**, 231: 115682. https://doi.org/10.1016/j.carbpol.2019.115682
[25] Fahrioğlu, U., Dodurga, Y., Elmas, L., et al. Ferulic acid decreases cell viability and colony formation while inhibiting migration of MIA PaCa-2 human pancreatic cancer cells *in vitro*. *Gene*, **2016**, 576: 476–482. https://doi.org/10.1016/j.gene.2015.10.061
[26] Dodurga, Y., Eroğlu, C., Seçme, M., et al. Anti-proliferative and anti-invasive effects of ferulic acid in TT medullary thyroid cancer cells interacting with URG4/URGCP. *Tumor Biology*, **2015**, 37: 1933–1940. https://doi.org/10.1007/s13277-015-3984-z
[27] Fong, Y., Tang, C. C., Hu, H. T., et al. Inhibitory effect of trans-ferulic acid on proliferation and migration of human lung cancer cells accompanied with increased endogenous reactive oxygen species and β-catenin instability. *Chinese Medicine*, **2016**, 11: 1–13. https://doi.org/10.1186/s13020-016-0116-7
[28] Kumar, N. V., Kanta, S. N., Madhusmita, S., et al. *In vitro* anticancer activity of *Eclipta alba* whole plant extract on colon cancer cell HCT-116. *BMC Complementary Medicine and Therapies*, **2020**, 20: 355. https://doi.org/10.1186/s12906-020-03118-9
[29] Canan, E., Mücahit, S., Gülseren, B., et al. Assessment of the anticancer mechanism of ferulic acid via cell cycle and apoptotic pathways in human prostate cancer cell lines. *Tumour Biology*, **2015**, 36: 9437–9446. https://doi.org/10.1007/s13277-015-3689-3
[30] Hou, Y. Z., Yang, J., Zhao, G. R., et al. Ferulic acid inhibits vascular smooth muscle cell proliferation induced by angiotensin II. *European Journal of Pharmacology*, **2004**, 499: 85–90. https://doi.org/10.1016/j.ejphar.2004.07.107
[31] Chang, C. J., Chiu, J. H., Tseng, L. M., et al. Modulation of HER2 expression by ferulic acid on human breast cancer MCF7 cells. *European Journal of Clinical Investigation*, **2006**, 36: 588–596. https://doi.org/10.1111/j.1365-2362.2006.01676.x
[32] Eun, C. Y., Eunmi, P. Ferulic acid in combination with PARP inhibitor sensitizes breast cancer cells as chemotherapeutic strategy. *Biochemical and Biophysical Research Communications*, **2015**, 458: 520–524. https://doi.org/10.1016/j.bbrc.2015.01.147
[33] Janicke, B., Hegardt, C., Krogh, M., et al. The antiproliferative effect of dietary fiber phenolic compounds ferulic acid and p-coumaric acid on the cell cycle of Caco-2 cells. *Nutrition and Cancer*, **2011**, 63: 611–622. https://doi.org/10.1080/01635581.2011.538486
[34] Karthikeyan, S., Kanimozhi, G., Prasad, N. R., et al. Radiosensitizing effect of ferulic acid on human cervical carcinoma cells *in vitro*. *Toxicology in Vitro*, **2011**, 25: 1366–1375. https://doi.org/10.1016/j.tiv.2011.05.007
[35] Ting, W., Xia, G., Rong, J., et al. Ferulic acid inhibits proliferation and promotes apoptosis via blockage of PI3K/Akt pathway in osteosarcoma cell. *American Journal of Translational Research*, **2016**, 8: 968–980.
[36] Muthusamy, G., Balupillai, A., Ramasamy, K., et al. Ferulic acid reverses ABCB1-mediated paclitaxel resistance in MDR cell lines. *European Journal of Pharmacology*, **2016**, 786: 194–203. https://doi.org/10.1016/j.ejphar.2016.05.023
[37] Nakanishi, M., Shimada, M., Niida, H. Genetic instability in cancer cells by impaired cell cycle checkpoints. *Cancer Science*, **2006**, 97: 984–989. https://doi.org/10.1111/j.1349-7006.2006.00289.x
[38] Gao, J., Yu, H., Guo, W., et al. The anticancer effects of ferulic acid is associated with induction of cell cycle arrest and autophagy in cervical cancer cells. *Cancer Cell International*, **2018**, 18: 1–9. https://doi.org/10.1186/s12935-018-0595-y
[39] Saenglee, S., Jogloy, S., Patanothai, A., et al. Cytotoxic effects of peanut phenolics possessing histone deacetylase inhibitory activity in breast and cervical cancer cell lines. *Pharmacological Reports*, **2016**, 68: 1102–1110. https://doi.org/10.1016/j.pharep.2016.06.017
[40] Reddy, B. V., Prasad, N. R. 2-Deoxy-*D*-glucose and ferulic acid modulates radiation response signaling in non-small cell lung cancer cells. *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine*, **2013**, 34: 251–259. https://doi.org/10.1007/s13277-012-0545-6
[41] Siavoshian, S., Blottiere, H. M., Cherbut, C., et al. Butyrate stimulates cyclin D and p21 and inhibits cyclin-dependent kinase 2 expression in HT-29 colonic epithelial cells. *Biochemical and Biophysical Research Communications*, **1997**, 239: 839–843. https://doi.org/10.1006/bbrc.1997.6255
[42] Litvak, D. A., Evers, B. M., Hwang, K. O., et al. Butyrate-induced differentiation of Caco-2 cells is associated with apoptosis and early induction of p21Waf1Cip1 and p27Kip1. *Gastroenterology*, **1998**, 115: 421–430. https://doi.org/10.1016/S0039-6060(98)70116-3
[43] Israels, E. D. The cell cycle. *Stem Cells*, **1995**, 19: 88–96. https://doi.org/10.1634/stemcells.19-1-88
[44] Resnitzky, D., Reed, S. I. Different roles for cyclins D1 and E in regulation of the G1-to-S transition. *Journal of Molecular Biology*, **1995**, 255: 3463–3469. https://doi.org/10.1128/MCB.15.7.3463
[45] Boonstra, J. Progression through the G1-phase of the on-going cell cycle. *Journal of Cellular Biochemistry*, **2003**, 90: 244–252. https://doi.org/10.1002/jcb.10617
[46] Kim, E. M., Jung, C. H., Kim, J., et al. The p53/p21 complex regulates cancer cell invasion and apoptosis by targeting Bcl-2 family proteins. *Cancer Research*, **2017**, 77: 3092–3100. https://doi.org/10.1158/0008-5472.CAN-16-2098
[47] Blackford, A. N., Jackson, S. P. ATM, ATR, and DNA-PK: the trinity at the heart of the DNA damage response. *Molecular Cell*, **2017**, 66: 801–817. https://doi.org/10.1016/j.molcel.2017.05.015
[48] Zhang, J., Gan, Y., Li, H., et al. Inhibition of the CDK2 and cyclin A complex leads to autophagic degradation of CDK2 in cancer cells. *Nature Communications*, **2022**, 13: 2835. https://doi.org/10.1038/s41467-022-30264-0
[49] Chandarlapaty, S., Razavi, P. Cyclin E mRNA: assessing cyclin-dependent kinase (CDK) activation state to elucidate breast cancer resistance to CDK4/6 inhibitors. *Journal of Clinical Oncology*, **2019**, 37: 1148–1150. https://doi.org/10.1200/JCO.19.00090
[50] Chen, S., Zhou, Q., Guo, Z., et al. Inhibition of MELK produces potential anti-tumour effects in bladder cancer by inducing G1/S cell cycle arrest via the ATM/CHK2/p53 pathway. *Journal of Cellular and Molecular Medicine*, **2020**, 24: 1804–1821. https://doi.org/10.1111/jcmm.14878
[51] Barnum, K. J., O'Connell, M. J. Cell cycle regulation by checkpoints. *Methods in Molecular Biology*, **2014**, 1170: 29–40. https://doi.org/10.1007/978-1-4939-0888-2_2
[52] Campos, A., Clemente-Blanco, A. Cell cycle and DNA repair regulation in the damage response: protein phosphatases take over the reins. *International Journal of Molecular Sciences*, **2020**, 21: 446–446. https://doi.org/10.3390/ijms21020446
[53] Ding, L., Cao, J., Lin, W., et al. The roles of cyclin-dependent kinases in cell-cycle progression and therapeutic strategies in human breast cancer. *International Journal of Molecular Sciences*, **2020**, 21: 1960. https://doi.org/10.3390/ijms21061960
[54] Bartek, J., Lukas, J. Cell-cycle checkpoints and cancer. *Nature*, **2003**, 421: 487–493. https://doi.org/10.1038/nature03097
[55] Stefanikova, A., Klacanova, K., Pilchova, I., et al. Cyclin-dependent kinase 2 inhibitor SU9516 increases sensitivity of colorectal carcinoma cells Caco-2 but not HT29 to BH3 mimetic ABT-737. *General Physiology and Biophysics*, **2017**, 36: 539–547. https://doi.org/10.4149/gpb_2017030