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Original Research

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The edible ethanol extract of *Rosa hybrida* suppresses colon cancer progression by inhibiting the proliferation-cell signaling-metastasis axis

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ABSTRACT

BACKGROUND/OBJECTIVES: *Rosa hybrida* has been demonstrated to exert biological effects on several cell types. This study investigated the efficacy of the edible ethanol extract of *R. hybrida* (EERH) against human colorectal carcinoma cell line (HCT116) cells.
MATERIALS/METHODS: HCT116 cells were cultured with different concentrations of EERH (0, 400, 600, 800, and 1,000 μg/mL) in Dulbecco's modified Eagle medium. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide and viable cell counting assays. Cell cycle pattern was observed by flow cytometry analysis. The wound-healing migration assay, invasion assay, and zymography were used to determine the migratory and invasive level of HCT116 cells treated with EERH. The protein expression and binding ability level of HCT116 cells following EERH treatment were analyzed via immunoblotting and the electrophoretic mobility shift assay.
RESULTS: EERH suppressed HCT116 cell proliferation, thus arresting the G1-phase cell cycle. It also reduced cyclin-dependent kinases and cyclins, which are associated with p27KIP1 expression. Additionally, EERH differentially regulated the phosphorylation of extracellular signal-regulated kinase 1/2, c-Jun NH2-terminal kinase, p38, and protein kinase B. Moreover, EERH treatment inhibited the enzymatic activity of matrix metalloproteinase-9 (MMP-9) and MMP-2, resulting in HCT116 cell migration and invasion. The EERH-induced inhibition of MMP-9 and MMP-2 was attributed to the reduced transcriptional binding of activator protein-1, specificity protein-1, and nuclear factor-κB motifs in HCT116 cells. Kaempferol was identified as the main compound contributing to EERH's antitumor activity.
CONCLUSION: EERH inhibits HCT116 cell proliferation and metastatic potential. Therefore, it is potentially useful as a preventive and curative nutraceutical agent against colorectal cancer.

Keywords: HCT116 cells; cyclin-dependent kinases; cyclins; metalloproteases

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Conflict of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Kim HM, Lee D, Moon SK; Formal analysis: Kim HM, Lee D, Song JH, Kim H, Lee S, Shin S; Investigation: Song JH, Kim H, Lee S, Shin S; Methodology: Kim HM, Lee D; Project administration: Moon SK; Supervision: Park SD, Kim YW, Choi YH, Kim WJ, Moon SK; Validation: Kim HM, Lee D, Song JH, Kim H, Lee S, Shin S, Park SD, Kim YW, Choi YH, Kim WJ; Writing - original draft: Kim HM, Lee D, Moon SK; Writing - review & editing: Kim HM, Lee D, Moon SK.

INTRODUCTION

Colorectal cancer is the third most common and second leading cause of mortality related to malignant carcinoma worldwide, with adenocarcinoma originating from the colonic and rectal epithelia accounting for approximately 90% of cases. The incident rates in developed countries are approximately 4 times higher than those in transitioning countries [1,2]. While surgical resection remains the optimal treatment, it is applicable to only 20–25% of stage I and II patients. Post-operative recurrence rates range from 40% to 70%. Palliative chemotherapy becomes necessary for generally inoperable, recurrent, and metastatic cases, with adjuvant chemotherapy recommended for stage III post-surgery [3,4]. Notably, liver metastasis is a primary cause of death in colorectal cancer, yielding a mortality rate exceeding 70% [5]. Stage II and III cancers, exhibiting p27KIP1 loss or loss of heterozygosity at chromosomes 5q, 17p, and 18q, demonstrate dismal prognosis [3,4]. Recent advancements in chemotherapy have improved patient survival; however, stage IV remains largely incurable [6,7]. For research into cancer biology, colon cancer, and liver metastasis, the human colorectal carcinoma cell line (HCT116) serves as an ideal model [8,9].

Eukaryotic mitosis comprises 4 phases: G1, G2, S, and M, each regulated by cell-cycle checkpoints. Cell cycle inhibitors, cyclin-dependent kinases (CDKs), and cyclins act as cell-cycle regulators, modulating various stages of signal cascades [10-12]. The DNA damage response suppresses individual cyclin/CDK complex activity via CDK inhibitors (CDKIs), impeding cell cycle progression [8,9]. CDKIs, including p21WAF1 and p27KIP1, block G1- to S-phase progression, inhibiting the kinase activities of cyclin/CDK complexes [10-12]. Interestingly, conflicting results suggest that p21WAF1 also plays influential, supportive, and proliferative roles in activating cyclin/CDK [13]. Poor prognoses are observed in patients with colorectal cancer lacking or with reduced p27KIP1 levels [14]. Apart from p27KIP1, superior molecular markers for colon cancer-specific or overall mortality have not yet been identified [15]. Therefore, stabilizing p27KIP1 potentially prevents the progression of precancerous adenoma into malignant carcinomas [16].

Mismatch-repair defects are prevalent in colorectal cancer, potentially attenuating p53 pathway activity [6]. Wild-type p53, when activated by multiple cellular stresses, orchestrates cell-cycle arrest and acts as a cell-death checkpoint [17]. Among the numerous biological processes, such as cell proliferation, inflammation, migration, differentiation, and invasion, central cascades include the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways [18]. Notably, RAS and BRAF oncogenic mutations, occurring in 37% and 13% of colorectal cancers, respectively, activate the MAPK and PI3K/AKT signaling pathways [18,19].

Matrix metalloproteinase-9 (MMP-9) and MMP-2, zinc-dependent endopeptidases, facilitate tumor-cell degradation into extracellular matrix (ECM) components during migration and invasion [20]. MMP-9 and MMP-2 promote HCT116 cell migration and invasion by degrading type IV collagen, potentially causing epithelial cell instability [21,22]. The abundance of MMP-9 and MMP-2 in tissue and serum correlates with muscle-invasive disease [20,23]. Transcription factors, such as specificity protein 1 (Sp-1), activator protein 1 (AP-1), and nuclear factor kappa B (NF-κB), control MMP-9 and MMP-2 expression at promoter regions [20,23]. Consequently, the repression of MMP-9 and MMP-2 expression is a potentially crucial process in preventing cellular migration and invasion [23,24].

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Most current anti-cancer molecules affect essential functions in both normal and cancer cells [25]. Discovering agents that specifically target cancer cells while remaining safe and well tolerated in patients has been an ongoing endeavor in oncology. Natural products derived from edible plants have gained attention owing to their potential as effective and safer alternatives in tumor prevention or treatment [26]. Edible *Rosa hybrida*, a renowned source of aromatic oils and physiological components in the nutraceutical and cosmetic industries, predominantly comprises gallic acid and volatiles, such as 1-butanol, cyclododecane, and dodecyl acrylate. Its extracts, recognized for their anti-inflammatory, antimicrobial, antioxidant, and neuroprotective properties, exert diverse nutritional and physiological effects [27,28]. Our previous study revealed the preventive role of the water extract of *R. hybrida* against the proliferation and migration of platelet-derived growth factor-stimulated vascular smooth muscle cells [29]. Furthermore, we identified kaempferol as the major active compound in the ethanol extract of *R. hybrida* (EERH) through nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis, which was confirmed by high-performance liquid chromatography (HPLC). This compound demonstrated significant antitumor effects in bladder cancer T24 cells [30]. Building on these findings, our study investigated the effects of EERH on HCT116 cell proliferation, migration, and invasion.

MATERIALS AND METHODS

Materials

Antibodies against p38MAPK (#9212), phospho-p38MAPK (#9211), c-Jun NH2-terminal kinase (JNK; #9258), phospho-JNK (#9251), extracellular signal-regulated kinases1/2 (ERK; #9102), phospho-ERK (#9101), AKT (#9272), phospho-AKT (#9271), normal rabbit immunoglobulin G (IgG; #2729S), and mouse IgG Isotype control (#5415S) were acquired from Cell Signaling (Danvers, MA, USA). Anti-CDK4 (sc-23896), cyclin D1 (sc-8396), CDK2 (sc-6248), cyclin E (sc-247), p21WAF1 (sc-6246), p27KIP1 (sc-1641), and p53 (sc-126) antibodies as well as glyceraldehyde 3-phosphate dehydrogenase (sc-47724) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-MMP-9 (#04-1150) and anti-MMP-2 (#AB19167) antibodies were secured from Millipore (Burlington, MA, USA). U0126, SB203580, LY294002, and SP600125 were purchased from Calbiochem (San Diego, CA, USA). Electrophoretic mobility shift assay (EMSA) (AY1XXX) and nuclear extraction kits were procured from Panomics (Fremont, CA, USA).

Preparation of the EERH

R. hybrida flowers were purchased from the Agricultural Technology Center (Jincheon, Korea). Dried petals (1.96 kg) were extracted using 50% ethanol for 3 h at 80°C. After filtering, the extract was concentrated under reduced pressure. Finally, 62.39 g of the ethanol extract was obtained and weighed.

Cell culture

The HCT116 and fetal human cell (FHC) cell lines were acquired from the American Type Culture Collection (Manassas, VA, USA). Both cell culture media comprised Dulbecco's modified Eagle medium (Sigma-Aldrich, San Diego, CA, USA) supplemented with 100 μg/mL streptomycin, 10% fetal bovine serum (FBS; 35-010-CV, Corning, NY, USA), and 100 U/mL penicillin. The cells were maintained at 37°C in a humidified incubator with 5% CO_2.

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Cell viability: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A concentration of 6×10^3 cells per well was cultivated in 96-well plates. The cells were incubated with EERH, and MTT solution (0.5 mg/mL, Sigma-Aldrich) was subsequently added to the cells for 1 h. After removing the solution, dimethyl sulfoxide was added, and the samples' absorbance values at 540 nm were determined using a fluorescent plate reader.

Viable cell counting assay

Cell viability values were expressed as percentages of the control group (untreated group). Cells were detached using a solution containing 0.25% trypsin–0.2% ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Waltham, MA, USA). Cell morphology was visualized using a phase-contrast microscope. The cells were reacted with 0.4% trypan blue (Sigma-Aldrich) and counted using a hemocytometer.

Cell cycle analysis

After treatment with EERH, cells were trypsinized, washed, and fixed in ethanol (70%) at −20°C for 24 h. The cells were washed several times with ice-cold phosphate-buffered saline (PBS) and subsequently incubated with a buffer containing 1 mg/mL RNase A and 50 mg/mL propidium iodide for the cell cycle assay. Using a Muse® Cell Analyzer equipped with analysis software (Merck Millipore, Darmstadt, Germany), cell-cycle phases were distributed and quantified.

Immunoblotting and immunoprecipitation

Cells were washed 2–3 times with ice-cold PBS and resuspended in lysis buffer, which comprised 150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5), 1 mM EDTA, 2.5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Tween-20, 2 μg/mL aprotinin, and 10 μg/mL leupeptin at 4°C for 30 min. Thereafter, the cells were collected and stored on ice for 10 min. The extracts were subsequently centrifuged at 12,000 × g and 4°C for 15 min. The total protein concentration of the cell lysates was quantified using the bicinchoninic acid protein assay reagent kit (Thermo Fisher Scientific). The total proteins (25 μg) were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and subsequently transferred to nitrocellulose membranes (Hybond®; GE Healthcare Bio-Sciences, Marlborough, MA, USA). A 5% skim milk solution was used to block the membrane for 2 h. Thereafter, the membrane was incubated with primary antibodies and subsequently reacted with peroxidase-conjugated secondary antibodies. To detect the protein bands, an electrochemiluminescence kit (GE Healthcare Bio-Sciences) was employed. For immunoprecipitation, the proteins (200 μg) from the cell lysates were added to the indicated antibodies and incubated at 4°C overnight. Protein A Sepharose® beads (sc2003, Santa Cruz) were incubated with the mixture at 4°C for 2 h. Final immunoprecipitation samples were collected and rinsed, and an equal amount of protein was utilized for SDS–PAGE separation, followed by immunoblotting.

Wound-healing migration assay

Cells (3×10^5/well) were incubated in 6-well plates. A 2-mm-wide pipette tip was used to scratch the cellular surface. Thereafter, the cells were washed and cultured in fresh medium supplemented with various EERH concentrations for 24 h. Subsequently, the width of the wound area was photographed and determined by quantitating cell migration into the scratched region using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

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Invasion assay

Cells (2.5×10^4/well) were implanted and incubated in a serum-free culture medium supplemented with various EERH concentrations for 2 h in the upper chamber of the incorporated Transwell™ insert with 8-μm polycarbonate pores (Sigma-Aldrich). Thereafter, culture medium containing 10% FBS was added and kept at 37°C in the lower chamber. After 24-h incubation, the cells invading the lower chamber were washed, fixed in ethanol for 30 min, and subsequently stained with 0.1% crystal violet in 20% ethanol for 30 min. Photographs were taken and cells counted. The percentage of invading cells was compared with those in the control group.

Zymography

MMP-9 and MMP-2 activities were evaluated using the gelatin zymographic assay. Cells were implanted and treated with various EERH concentrations for 24 h. Thereafter, the conditioned medium was collected, and electrophoresis was performed on a 0.25% gelatin–polyacrylamide gel. Prior to incubation with renaturing buffer (150 mM NaCl, 50 mM Tris-HCl, and 10 mM $CaCl_2$; pH 7.5) at 37°C overnight, the gel was rinsed with 2.5% Triton X-100 solution for 15 min at room temperature. Finally, the gel was stained with 0.2% Coomassie blue to visualize gelatinase activities using a light box. White bands were visualized against a blue background and measured as MMP-9 and MMP-2 gelatinase activities.

EMSA

The nuclear proteins in EERH-treated cells were extracted using the nuclear extraction kit (AY2002, Panomics). After centrifugation, the cells were rinsed and incubated on ice for 15 min in a buffer (10 mM KCl, 10 mM HEPES [pH 7.9], 1 mM DTT, 0.1 mM EGTA, 0.5 mM PMSF, and 0.1 mM EDTA). Subsequently, the cells were reacted with 0.5% NP-40 and centrifuged to obtain a nuclear pellet. Finally, the nuclear extracts were extracted via incubation at 4°C for 15 min using a buffer containing 400 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM PMSF, 1 mM EGTA, 1 mM DTT, and 1 mM EDTA.

Samples (10–20 μg) were blended with 100-fold excess unlabeled oligonucleotides at 4°C for 30 min. In this study, sequences were prepared as follows: AP-1, CTGACCCCTGA GTCAGCACT T; Sp-1, GCCCAT TCCTTCCGCCCCCAGATGAAGCAG; and NF-κB, CAG TGGAATT CCCCAGCC. The solutions were subsequently incubated at 4°C for 20 min with a buffer (25 mM HEPES buffer [pH 7.9], 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 2.5% glycerol) comprising poly dI/dC (2 μg) and 5 fmol (2×10^4 cpm) from a Klenow end-labeled (^{32}P adenosine triphosphate) 30-mer oligonucleotide, thus expanding the DNA-binding element of the MMP-9 and MMP-2 promoter. A 6% PAGE system at 4°C was used to separate the reaction solution. After exposing the gel to an X-ray film, the values of the gray blots were visually evaluated using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

HPLC-ultraviolet (UV) analysis of kaempferol from EERH

Samples were prepared for HPLC analysis by dissolving 10 to 20 mg of the petal extracts in 1 mL MeOH. A standard stock solution was prepared by dissolving 1 mg kaempferol in 1 mL MeOH. All samples were filtered through a 0.45-μm polyvinylidene fluoride filter prior to use. An HPLC system was used, and chromatographic separation was achieved using an INNO C18 column (250 mm × 4.6 mm, 5 μm; Young Jin Biochrom Co., Ltd., Seongnam, Korea). A gradient elution of 0.5% acetic acid in water and methanol was used (0–30 min: 50% methanol, 30–45 min: 50% methanol, and 45–60 min: 50–10% methanol). The flowrate and injection volume were 1 mL/min and 10 μL, respectively. The UV detector was set at 270 nm.

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Statistical analysis

Each experiment was performed in triplicate, and all data are expressed as the mean ± SD. The data were analyzed using student's *t*-test, and statistical significance was set at $P < 0.05$.

RESULTS

EERH inhibited the proliferation of HCT116 cells by arresting the G0/G1 phase of the cell cycle

MTT assay and cell counting data demonstrated a dose-dependent decrease in HCT116 cell growth upon EERH treatment for 24 h (**Fig. 1A and B**). This decline in cell growth was consistent across various EERH concentrations (0, 400, 600, 800, and 1,000 μg/mL), as

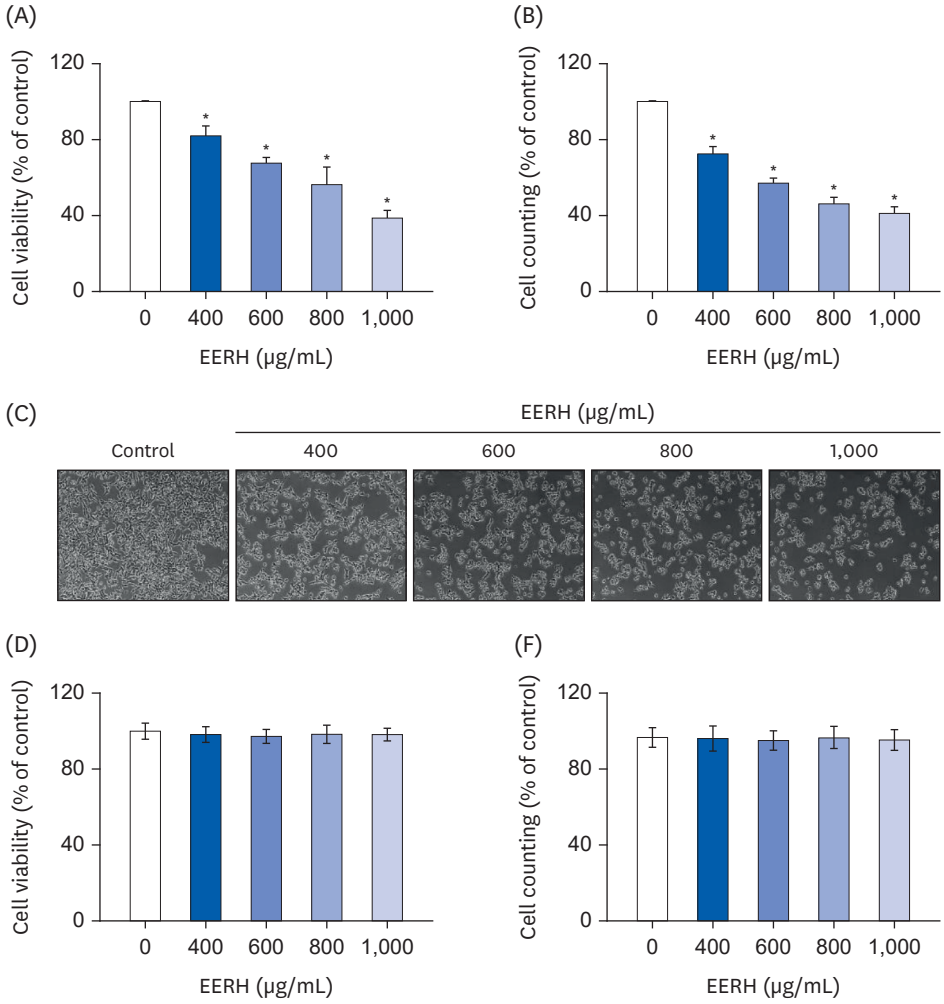

Fig. 1. Effect of EERH on the proliferation of human colon cancer HCT116 cells and their morphology. HCT116 cells were treated with or without EERH under various concentrations (0, 400, 600, 800, and 1,000 μg/mL) for 24 h. (A) Cell viability was determined using the MTT assay. (B) Cell counts were performed using a hemocytometer and microscope. (C) The morphological changes of HCT116 cells were imaged using an inverted microscope (×40 magnification). (D, E) MTT and cell counting assay were performed in EERH-treated FHC colon normal cells. Values are presented as the mean ± SD of 3 independent experiments (*) compared with the control.
EERH, ethanol extract of *Rosa hybrida*; HCT116, human colorectal carcinoma cell line; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FHC, fetal human cell.
*$P < 0.05$.

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depicted in **Fig. 1A and B**. Similar results were confirmed for cell proliferation via microscopic examination (**Fig. 1C**). Additionally, after 48 h of EERH treatment, the cell growth at 1,000 μg/mL concentration decreased by approximately 20% (**Supplementary Fig. 1**). In contrast, EERH treatment did not significantly affect cell growth in normal colon epithelial FHC cells, suggesting that EERH selectively inhibits the proliferation of cancer cells (**Fig. 1D and E**). Flow cytometry revealed an increased proportion of cells with EERH-induced G0/G1 phase arrest compared with that in the control group (**Fig. 2**). This effect was notably observed at EERH concentrations ranging from 400 to 1,000 μg/mL (**Fig. 2**).

EERH induced G0/G1 phase arrest via cyclin/CDK complexes downregulation and p27KIP1 upregulation

Further investigation into the mechanisms underlying EERH-induced G0/G1 arrest focused on cell-cycle protein regulation during the G0/G1 phase. CDK4 and CyclinD1 protein expression levels significantly decreased upon EERH treatment (**Fig. 3A**). Additionally, p27KIP1 protein expression exhibited a marked increase compared with that of p21WAF1 and p53 after EERH treatment (**Fig. 3B**). Notably, the increased expression of p27KIP1 responded to EERH treatment dose-dependently, suggesting the crucial role of p27KIP1 as a cell-cycle inhibitor (**Fig. 3B**). Interestingly, p21WAF1 and p53 expression levels did not appear to relate to EERH-mediated G1-phase cell cycle arrest (**Fig. 2**). Further investigation revealed a considerable increase in CDK4/p27KIP1 complex formation in EERH-treated cells (**Fig. 3C**). Collectively, these findings suggest that EERH-induced G1-phase cell cycle arrest in HCT116 cells is associated with CDK4 and cyclin D1 downregulation via upregulation of p27KIP1.

EERH induced the phosphorylation of ERK, JNK, and AKT and inhibited the phosphorylation of p38

We subsequently performed the immunoblot experiment to identify the regulatory signaling pathways in EERH-treated HCT116 cells. In HCT116 cells, EERH induced the phosphorylation of ERK, JNK, and AKT but suppressed that of p38 (**Fig. 4A**). The EERH-induced phosphorylation of JNK, ERK, and AKT was confirmed using specific kinase inhibitors (U0126, SP600125, and LY294002) (**Fig. 4B**). The results revealed that EERH potentially

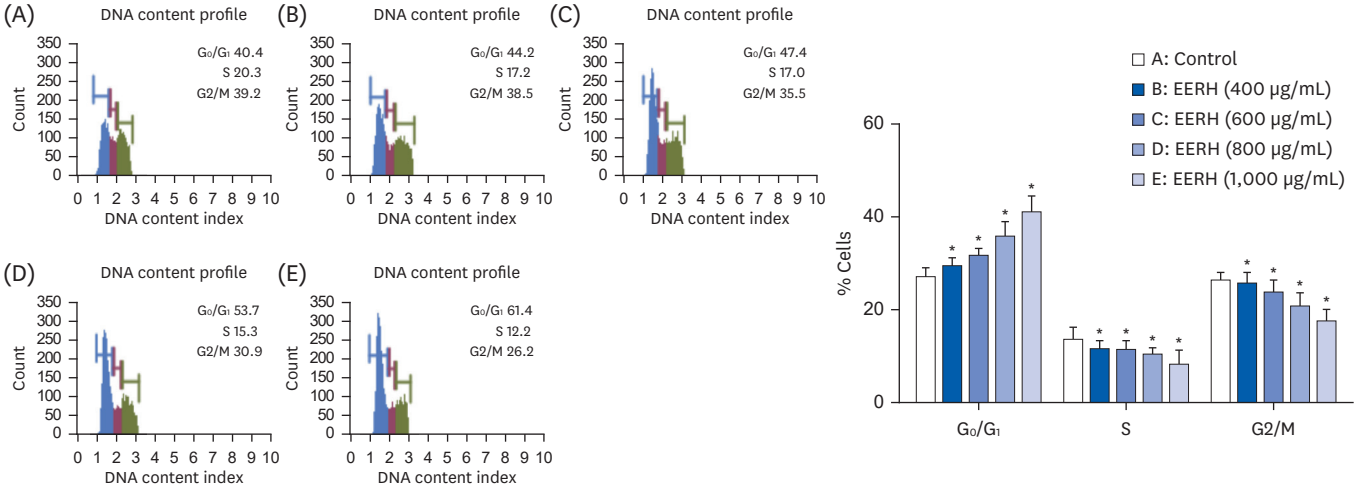

Fig. 2. EERH induced G_0/G_1-phase arrest in human colon cancer HCT116 cells. Cells were treated with (A) 0, (B) 400, (C) 600, (D) 800, and (E) 1,000 μg/mL of EERH for 24 h. Flow cytometry analysis was performed to determine the effect of EERH on the cell cycle. The percentage of cells in each phase is displayed as the mean ± SD of 3 independent experiments (*).
EERH, ethanol extract of *Rosa hybrida*; G_0/G_1, gap 0/gap 1; HCT116, human colorectal carcinoma cell line.
*$P < 0.05$.

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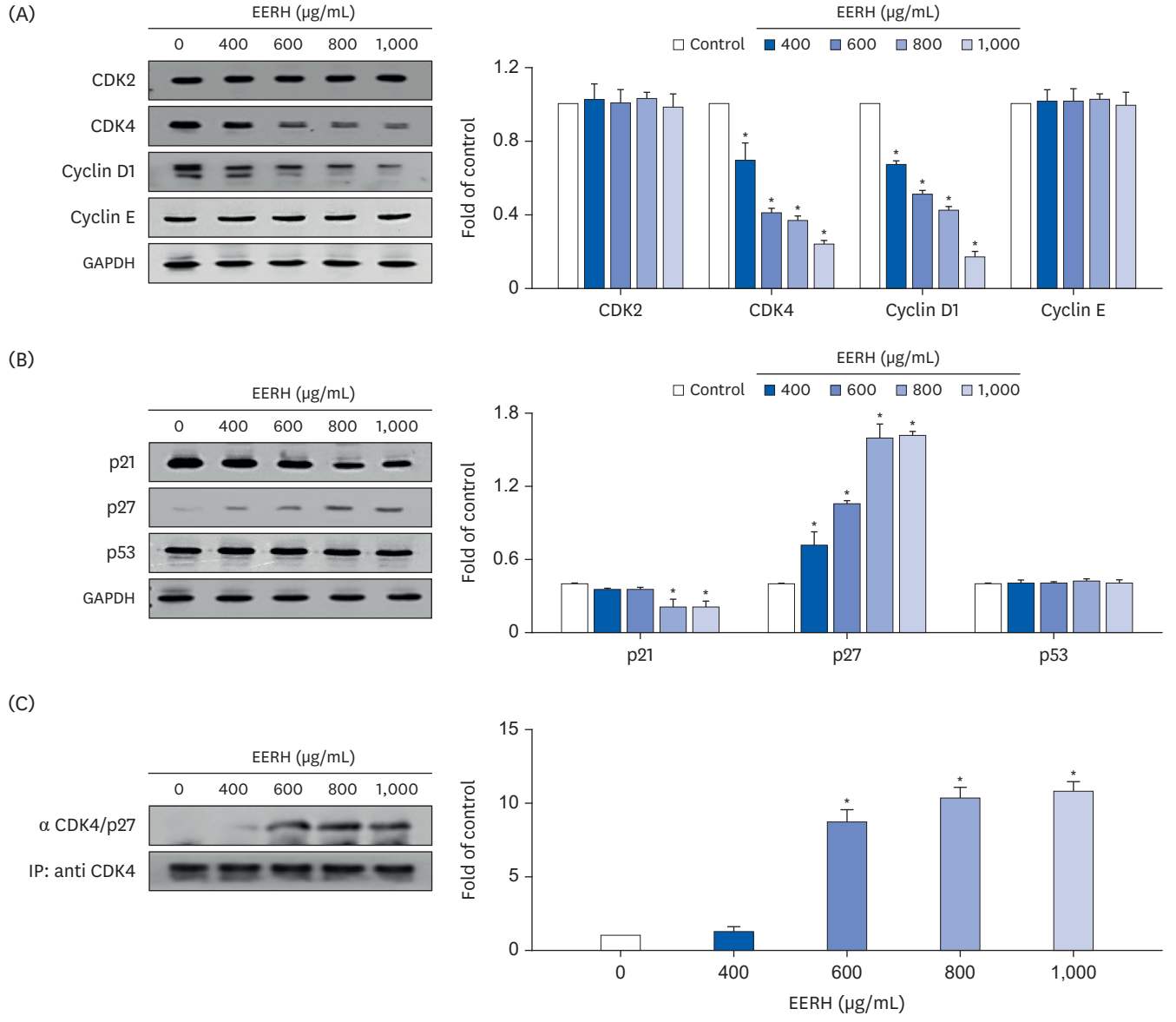

Fig. 3. EERH altered the expression levels of G_0/G_1-phase cell cycle-related proteins for 24 h. EERH induced G_0/G_1-phase cell-cycle arrest by decreasing CDK4 and cyclin D1 expression and increasing p27KIP1 expression. (A, B) HCT116 cells were treated with various concentrations (0, 400, 600, 800, and 1,000 μg/mL) of EERH for 24 h. Immunoblot analysis was performed using individual antibodies. GAPDH was used as a loading control. (C) IP was confirmed using an anti-CDK4 antibody, followed by immunoblotting with p27KIP1. CDK4 immunoprecipitation was performed using an anti-CDK4 antibody. Values are presented as the mean ± SD of 3 independent experiments (*) compared with the control.
EERH, ethanol extract of *Rosa hybrida*; G_0/G_1, gap 0/gap 1; CDK, cyclin-dependent kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IP, immunoprecipitation; HCT116, human colorectal carcinoma cell line; p27KIP1, cyclin-dependent kinase inhibitor 1B.
*$P < 0.05$.

stimulated different interconnected signaling pathways during the EERH-stimulated inhibition of HCT116 cell proliferation (**Fig. 4B**). These findings suggest that EERH modulates cell growth by regulating the p38, JNK, ERK, and AKT pathways.

EERH mitigated the migratory and invasive potential of HCT116 cells

Migration and invasion of HCT116 cells are critical factors in tumor progression, often leading to cancer metastasis to distant organs, such as the liver [25,26]. To assess the effect of EERH on migratory and invasive processes, wound-healing and invasion assays were

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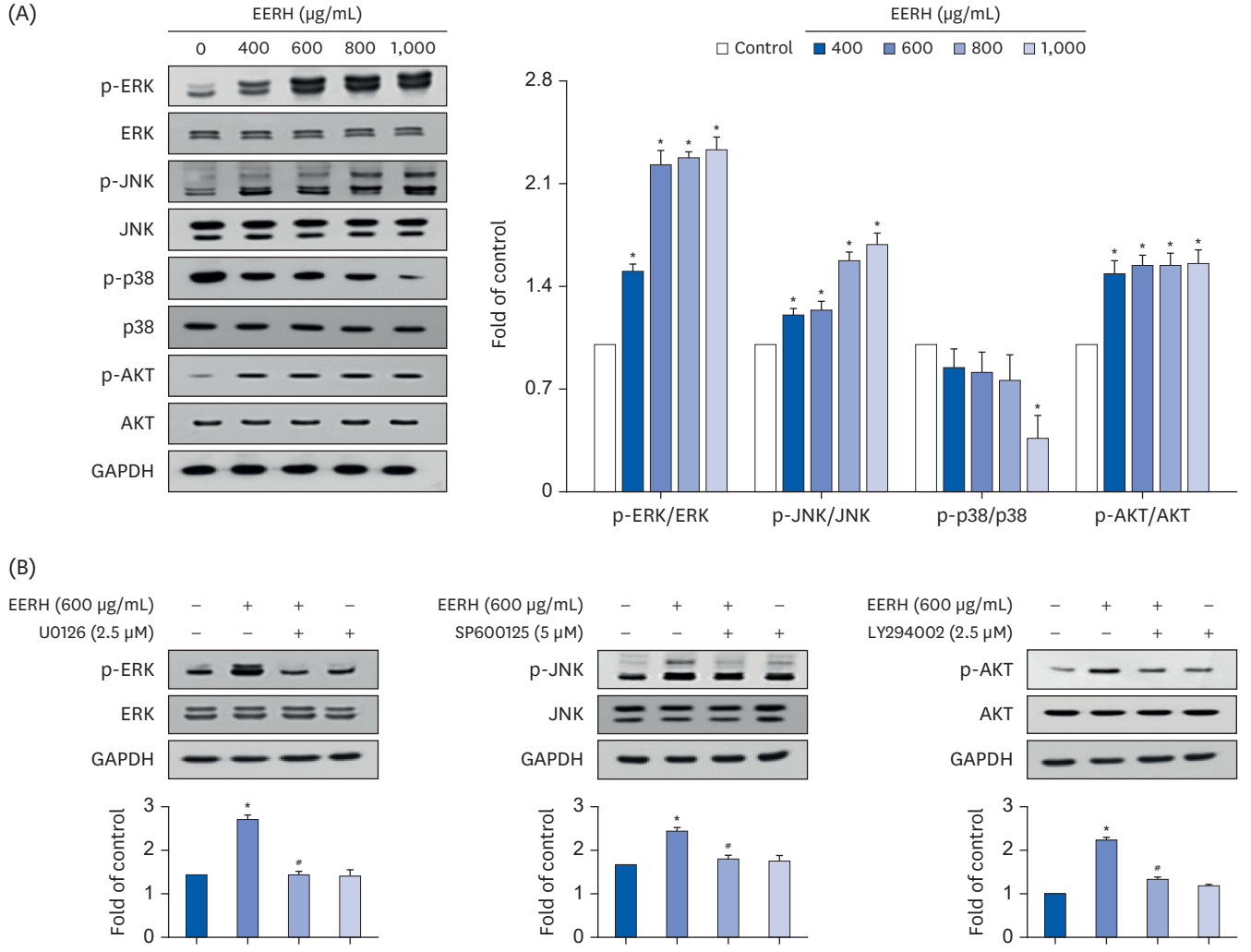

Fig. 4. EERH induced ERK, JNK, and AKT phosphorylation in human colon cancer HCT116 cells for 24 h. HCT116 cells were treated with various concentrations of EERH (0, 400, 600, 800, and 1,000 μg/mL) for 24 h. (A) EERH increased the phosphorylation of ERK and JNK and decreased that of p38 MAPK in HCT116 cells for 24 h. EERH increased AKT phosphorylation in HCT116 cells for 24 h. (B) HCT116 cells were incubated with specific inhibitors: U0126 (2.5 μM), SP600125 (10 μM), and LY294002 (2.5 μM) for ERK, JNK, and AKT, respectively, for 30 min, followed by treatment with EERH (600 μg/mL). Immunoblotting was performed using indicated antibodies against specific antibodies. GAPDH was used as an internal control. Values are presented as the mean ± SD of 3 independent experiments compared with the control (*) or EERH treatment (#).
EERH, ethanol extract of *Rosa hybrida*; ERK, extracellular signal-regulated kinases1/2; JNK, c-Jun N-terminal kinases; AKT, protein kinase B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; p38 MAPK, p38 mitogen-activated protein kinases; HCT116, human colorectal carcinoma cell line; U0126, MEK inhibitor; SP600125, JNK inhibitor; LY294002, AKT inhibitor.
*$P < 0.05$; #$P < 0.05$.

conducted. EERH treatment significantly reduced the migratory capacity of HCT116 cells (**Fig. 5A**). In addition, HCT116 cell invasiveness was notably hindered in the presence of EERH (**Fig. 5B**). These findings demonstrate that EERH effectively inhibits the migratory and invasive capability of HCT116 cells, potentially impeding tumor progression and metastasis.

EERH inhibited MMP-9 and MMP-2 activity by abolishing the binding capacities of transcriptional factors (Sp-1, AP-1, and NF-κB) in HCT116 cells

MMP-9 and MMP-2 expression levels serve essential roles in HCT116 cell migration and invasion [25]. Therefore, EERH's inhibitory effect on MMP-9 and MMP-2 expression levels in HCT116 cells was investigated using the gelatin zymographic assay. HCT116 cell treatment with EERH significantly attenuated MMP-9 and MMP-2 activity in a dose-dependent manner

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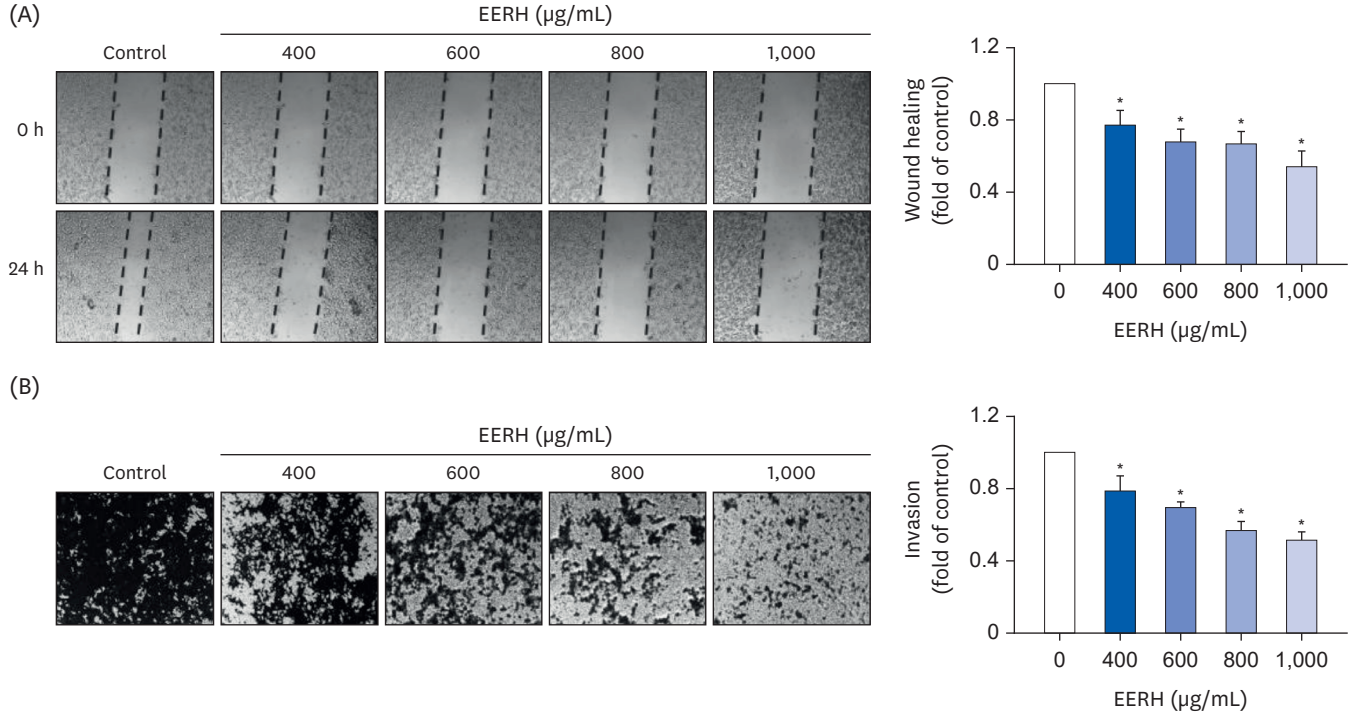

Fig. 5. Effect of EERH on the wound healing migration and invasion of human colon cancer HCT116 cells. (A) Cells (90% confluence) were scratched using a 10 μL-pipette tip and washed twice with PBS to remove the medium. The cells were subsequently treated with EERH (0, 400, 600, 800, and 1,000 μg/mL) for 24 h. Migration distance was measured using photomicrographs (×40 magnification). (B) The cells were seeded to the upper chamber of a gelatin-coated well and incubated with EERH (0, 400, 600, 800, and 1,000 μg/mL) for 24 h. Cells invading the lower surface of the membrane were captured using a microscope (×40 magnification). Values are designated as the mean ± SD of 3 independent experiments (*) compared with the control.
EERH, ethanol extract of *Rosa hybrida*; HCT116, human colorectal carcinoma cell line; PBS, phosphate-buffered saline.
*$P < 0.05$.

(**Fig. 6A**). To further elucidate the molecular mechanism underlying EERH's effect on MMP regulation, an EMSA was conducted using nuclear extracts. Briefly, the binding affinities of the AP-1, Sp-1, and NF-κB motifs, which are responsible for MMP-9 and MMP-2 expression, were assessed. EERH strongly suppressed binding abilities to the AP-1, Sp-1, and NF-κB motifs in HCT116 cells (**Fig. 6B**). These results indicate that EERH prevented MMP-9 and MMP-2 expression via the repressive binding activities of the transcription factors Sp-1, AP-1, and NF-κB in HCT116 cells (**Fig. 6B**).

EERH fraction contains kaempferol as the key bioactive compound

The presence of bioactive compound was analyzed through HPLC method (**Supplementary Fig. 2A**). The EERH fractions were investigated to identify the bioactive components responsible for their effects on HCT116 cell proliferation. Among the fractions, the d-fraction exhibited the strongest activity (**Supplementary Fig. 2B and C**) Comprehensive NMR and MS analyses identified kaempferol as the predominant bioactive compound in the d-fraction [30]. Our results confirmed that kaempferol was the main component responsible for the biological activity observed in HCT116 cells.

DISCUSSION

R. hybrida, utilized in the nutraceutical and cosmetic industries [27], exhibits diverse biological properties, such as antioxidant, anti-inflammatory, antimicrobial, and

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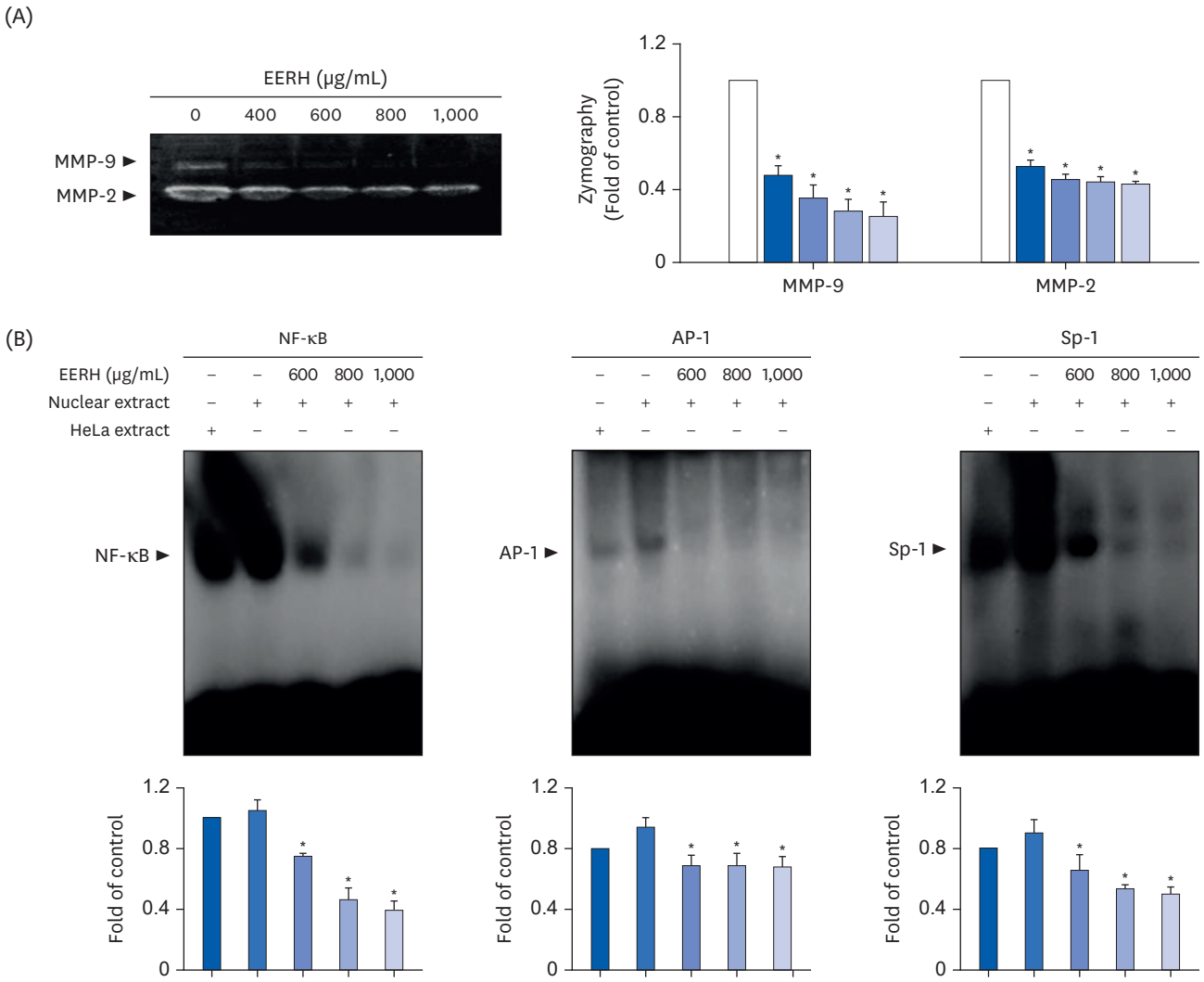

Fig. 6. EERH suppressed expression levels of MMP-9 and MMP-2 by inhibiting the binding activities of the transcription factors NF-κB, AP-1, and Sp-1 in human colon cancer HCT116 cells. (A) Cells were treated with EERH (0, 400, 600, 800, and 1,000 μg/mL) for 24 h. The supernatants of indicated cells were employed to examine MMP-2 and MMP-9 activities using gelatin zymography. (B) Nuclear extracts were collected from the cells and executed EMSA to evaluate the binding activities of NF-κB, AP-1, and Sp-1. Values are dipicted as the mean ± SD of 3 independent experiments (*) compared with the control.
EERH, ethanol extract of *Rosa hybrida*; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa B; AP-1, activator protein 1; Sp-1, specificity protein 1; HCT116, human colorectal carcinoma cell line; EMSA, electrophoretic mobility shift assay.
*$P < 0.05$.

neuroprotective activities [27,28]. This study is the first to ascertain whether *R. hybrida* extract exerts suppressive molecular effects on the proliferative, migratory, and invasive activities of HCT116 cells as a colorectal cancer model.

A key cellular hallmark of tumors is uncontrolled cell-cycle regulation, resulting in aberrant cell proliferation [31,32]. Several anti-cancer agents arrest specific phases of the cell cycle [33]. Therefore, controlling proteins that suppress specific cell-cycle phases is a potentially valuable anti-tumor treatment strategy [33,34]. G1-to-S transition during the mammalian cell cycle is regulated by CDK/cyclin complexes [35]. Our findings demonstrate that EERH induced cell-cycle arrest at the G1 phase in HCT116 cells, suppressing cyclin and CDK expression. Specifically, EERH significantly reduced the protein expression of CDK4 and its respective binding partner, cyclin D1. The cell cycle is coordinated by CDKIs, cyclins, and

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CDKs [31,32,36]. CDKIs, such as p27KIP1, bind to cyclin–CDK complexes, downregulating cell-cycle progression and inhibiting their kinase activities [37]. Clinical investigations have reported the loss of p27KIP1 expression and cytoplasmic localization of p27KIP1 in colon cancer [38], where a reduced p27KIP1 level promoted tumor development primarily driven by oncogenic events [16]. In the present study, EERH controlled the action mode of p27KIP1, but not that of p53, promoting the binding of p27KIP1 to CDK4, thereby suppressing G1-to-S cell-cycle phase transition. These results indicate that EERH-induced HCT116 cell inhibition involves arresting the G1 phase by upregulating p27KIP1.

MAPK and AKT signaling cascades play significant roles in various biological pathways, including proliferation, differentiation, migration, invasion, and inflammation, following growth factor treatment or stress stimulation [18,39]. MAPKs (p38 kinase, JNK, and ERK) and AKT possess established roles as early responsive kinases in regulating cancer cell proliferation [40-43]. The phosphorylation of p38, JNK, ERK, and AKT is closely associated with the inhibition of cell proliferation in several tumor cell lines [18,44]. The MAPK pathway is involved in the anti-apoptotic process, allowing colorectal cancer cells to survive, evade apoptosis, and develop chemo-resistance, often activated aberrantly in patients with colorectal cancer [39]. Recent studies have elucidated the critical roles of oncogenic mutations, such as KRAS and BRAF, which occur in approximately 37% and 13% of colorectal cancers, respectively [18,19]. These mutations lead to differential activation of downstream signaling pathways, including the ERK pathway. Specifically, BRAFV600E mutations are associated with robust and sustained ERK activation across various cellular contexts, whereas KRAS mutations, such as KRASG12V, exhibit a more selective ERK activation that is highly dependent on the cellular environment [45]. This selective activation results in diverse cellular outcomes, including reduced proliferation and migration. Our findings indicate that EERH affected the increase in JNK, AKT, and ERK phosphorylation and the decrease in p38 phosphorylation in HCT116 cells for 24 h. Studies have cumulatively demonstrated that the integration of multiple signaling pathways determines cell growth, proliferation, growth retardation, apoptosis, and differentiation in tumor progression [46]. Our findings suggest that EERH regulates the inhibition of HCT116 cell proliferation via differential multiple signaling pathways.

Wound healing migration and invasion experiments have been performed to determine whether EERH inhibits the metastatic potential of HCT116 cells [47]. Typically, elevated individual MMP levels strongly and positively correlated with advanced cancer stages in patients [48]. Both membrane-anchored and secreted MMPs [49] serve crucial roles in invasion and metastasis through their proteolytic activity [49]. Cancer invasion and migration constitute the initial step in metastasis, primarily mediated by type IV collagenases, such as MMP-9 (gelatinase B) and MMP-2 (gelatinase A) [47,50]. Metastasis involves tumor cells leaving a primary site, migrating to different body sites through the circulatory system, and forming malignant tumors [51]. The liver is a significant organ for colorectal cancer metastasis [23]. MMPs selectively degrade various ECM components and secrete cytokines and growth factors within the ECM [47,50]. Patients with colorectal cancer display high plasma levels of MMP-9 [47,50]. Bile from patients with metastasis possesses higher MMP-2 levels than that from those without metastasis [23]. Only invasive colorectal cancer, not a benign tumor, expresses MMP-2 [52]. Tumor cells may have a better survival rate within clusters than as individual cells in circulation [48]. The metastatic foci complex intrinsically includes transendothelial migration and invasion through the subendothelial basement membrane [48]. In our study, EERH treatment inhibited HCT116 cell migration

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and invasion, a process that co-occurred with a reduction in MMP-9 and MMP-2 enzymatic activity. A significant EERH-induced decrease in the wound-healing and invasive ability of HCT116 cells suggests EERH's potential for preventing and treating colorectal cancer. EERH potentially provides a supportive basis for the development of chemotherapeutic reagents for patients with colorectal cancer.

Numerous studies have suggested that the transcription factors AP-1, Sp-1, and NF-κB regulate MMP-2 and MMP-9 expression in tumor cells [28]. In addition, 3 major MAPKs, including the p38, JNK, and ERK pathways, regulate MMP-9 and MMP-2 expression [23]. Our study demonstrated that the inhibitory effect of EERH treatment on these transcription factors is responsible for reducing MMP-9 and MMP-2 activity. In colorectal cancer cells, MMP-9 and MMP-2 aid the metastasis and invasion of tumor cells to adjacent tissues or distant organs. Therefore, EERH treatment's inhibitory effect on MMP-9 and MMP-2 may be valuable in impeding tumor migration and invasion. These findings demonstrate that EERH prevented the migration and invasion of HCT116 cells via the suppression of MMP-9 and MMP-2 expression by inhibiting the binding activities of AP-1, Sp-1, and NF-κB motifs.

Kaempferol, a flavonoid compound found widely in edible plants and herbs, exhibits a range of biological activities including antioxidant, anti-inflammatory, and anticancer effects [53,54]. Recent studies have highlighted potential of kaempferol in antitumor activity across various cancers, such as non-small cell lung cancer, prostate cancer, and pancreatic tumors [55-58]. Kaempferol regulates key signaling pathways like AKT/PI3K and ERK, and inhibits the phosphorylation of TIMP2 and MMP-2 [56-58]. Additionally, kaempferol downregulates the expression of proteins such as Bcl-2, cyclin D1, and claudin-2. It also promotes apoptosis by upregulating pro-apoptotic factors, including PTEN, Bax, miR-340, and cleaved-caspases 3, 8, and 9 [56,57]. Kaempferol also induce ROS-dependent apoptosis in pancreatic cancer cells via TGM2-mediated AKT/mTOR signaling [58]. These multifaceted mechanisms underscore kaempferol's potential as a broad-spectrum anticancer agent, warranting further investigation into its therapeutic applications in various cancer models. In our study, the EERH was fractionated to identify the bioactive components responsible for its effects on HCT116 cells. The kaempferol present in the d-fraction exhibited the strongest activity in inhibiting cell proliferation. These findings emphasize the crucial role of kaempferol in mediating the antitumor effects of EERH, supporting its potential as a therapeutic agent across a variety of cancers.

In conclusion, our study elucidated the molecular mechanisms underlying the effects of EERH on HCT116 cells. Its findings suggest that EERH inhibits the proliferation of HCT116 cells via p27KIP1-mediated G1-phase cell-cycle arrest, attributed to a reduction in the cyclin D1/CDK4 complex. Additionally, HCT116 cell treatment with EERH affects the phosphorylation of the p38, JNK, ERK, and AKT signaling pathways. Furthermore, EERH treatment impedes HCT116 cell migration and invasion via diminished MMP-9 and MMP-2 expression by reducing the binding activities of AP-1, Sp-1, and NF-κB motifs. Chemical analysis identified kaempferol as the primary bioactive compound driving the antitumor effects observed in EERH. These findings further suggest that EERH holds promise in preventing and treating colorectal cancer. Further studies are required to investigate EERH's antitumor efficacy in animal models. Additional work should identify the active components of EERH available in colon cancer.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

EERH inhibited the proliferation of human colon cancer HCT116 cells after 48 h of treatment. The cells were treated with 0, 400, 600, 800, and 1,000 μg/mL of EERH for 48 h. MTT assay (A) and cell counting (B) were conducted to measure cell viability. Values are presented as the mean ± SD of 3 independent experiments (*) compared with the control.

Supplementary Fig. 2

Kaempferol identified as the bioactive compound in the EERH fractions. (A) HPLC analysis confirming the presence of kaempferol as the major component in the d-fraction. (B, C) The inhibitory effects of various EERH fractions on HCT116 cell proliferation were determined using the MTT assay and cell counting assay. Values are designated as the mean ± SD of 3 independent experiments (*) compared with the control.

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