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RESEARCH ARTICLE

Polyphenol galangin induces the ROS and ER stress-mediated intrinsic and extrinsic apoptotic pathways towards human colon carcinoma HCT-116 cells

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ABSTRACT

A natural anti-oxidant galangin, a flavonoid compound rich in the root of galangal, has been proved to exert anti-proliferation and apoptosis induction towards various cancer cells. However, its activities and molecular mechanism against colorectal cancer cell lines (e.g. HCT-116) is still unclear. The aim of this study was to reveal the inhibition and apoptosis induction of galangin in the HCT-116 cells. The results of CCK-8 assay demonstrated that galangin at 20-160 μmol/L inhibited the HCT-116 cells growth in a dose-time-dependent manner. Galangin changed cell morphology, arrested cell cycle at G_0/G_1 phase, decreased mitochondrial membrane potential, increased intracellular reactive oxygen species (ROS) and Ca^{2+} and induced early apoptosis in the HCT-116 cells. Based on western blotting results, on one hand, galangin at 80-160 μmol/L up-regulated pro-apoptotic proteins expression such as AIF, PIG3, and Bax, and induced mitochondrial pathway by up-regulating the levels of cleaved caspase-8, cleaved caspase-7, cleaved caspase-9 and cleaved caspase-3, as well as cleavage of poly (ADP-ribose) polymerase (PARP). On the other hand, galangin also induced endoplasmic reticulum (ER) stress in the cell via up-regulation of CHOP and DR5 and then activation of caspase cascades. The results illustrated that galangin induced apoptosis towards the HCT-116 cells through the ROS- and ER stress-mediated both intrinsic and extrinsic apoptotic pathways.

Keywords: Galangin; Human colon carcinoma cells; Apoptotic mechanism; Reactive oxygen species; Endoplasmic reticulum stress

INTRODUCTION

Cancer is the second worldwide cause of death, next to the cardiovascular illnesses. Current treatments such as surgery, radiotherapy, chemotherapy, hormone replacement therapy, immunotherapeutic interventions, targeted agents, growth signal inhibitors, endogenous angioinhibitors, gene therapy, stem cell therapy, and precision medicine are the main treatment strategies to patients (Akram et al., 2017; Singh et al., 2022). However, due to the short- and long-term side effects, innovation of alternative methods is being developed. For example, natural polyphenols are used as plant-derived bioactive medicines to deal with certain diseases (Singh et al., 2022). One class of these bioactive polyphenolic compounds is the flavonoids, the well-known secondary metabolites and natural anti-oxidants presented in plant kingdoms. Flavonoids can be chemically divided into these subclasses like isoflavanones, flavanones, flavanols, flavonols, flavones, and anthocyanidins. Galangin (3,5,7-trihydroxy-2-phenyl-4*H*-chromen-4-one), one member of the flavonoid family, are widely studied for its activities such as anti-oxidant, anti-metastatic, anti-microbial (Abinaya and Gayathri, 2019), anti-allergic inflammatory, anti-cancer, anti-diabetic, anti-osteoporosis, anti-obesity and anti-viral effects (Patel, 2015).

Galangin is presented in the root of *Alpinia officinarum* (Hance), *Helichrysum aureonitens, Alnus pendula Matsum, Betulaceae*, *Plantago major L*, *Plantaginaceae, Scutellaria galericulata L., Lamiaceae*, honey, and propolis (Patel, 2015). The present results have showed that galangin might be a potential candidate to suppress cell proliferation and induce cell apoptosis in various types of cancer cells including lung caner (Yu et al, 2018), gastric cancer (Xu et al, 2017),

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colorectal cancer (Wang et al, 2017), prostate cancer (Kazemi et al, 2021), ovarian cancer (Huang et al, 2015), head and neck cancers (Zhu et al, 2014), and osteosarcoma (Liu et al, 2017). For instance, it was found that 10-160 μmol/L of galangin could inhibit the proliferation of human breast cancer MCF-7 cells dose-dependently, down-regulate the cycle-associated proteins, induce cell apoptosis via mitochondrial pathway, and inhibit the phosphorylation of PI3K/Akt (Liu et al., 2018). It was also reported that galangin could inhibit the growth of several human liver cancer cell lines (HepG2, Hep3B and PLC/PRF/5) and cause cell apoptosis through the mitochondrial signaling pathway by corrupting the permeability of mitochondrial membrane and up-regulating the expression of these pro-apoptotic proteins such as Bax, cytochrome C and AIF (Zhang et al., 2010). Additionally, galangin could inhibit the growth of human osteosarcoma MG63 and U20S cells or intrahepatic cholangiocarcinoma HCCC9810 and TFK-1 cells, suppress cell migration and invasion, and enhance cell apoptosis via the PI3K and Aktp-Thr308 down-regulation (Yang et al., 2017; Zou et al., 2020). Interestingly, galangin may induce cell apoptosis via the endoplasmic reticulum (ER) stress pathway. For example, it was observed that galangin induced the ER stress-mediated apoptosis in hepatocellular carcinoma HepG2, Hep3B and PLC/PRF/5 cells, by up-regulating these indicator proteins of ER stress namely GRP94, GRP78, and CHOP, and by increasing free cytosolic Ca^{2+} level (Su et al., 2013). Galangin could stimulate ER stress in human breast cancer cells via promoting CHOP and DR5 expression, and cause cell apoptosis by activating TRAIL/caspase-3/AMPK signaling pathway (Song et al, 2017). Xiong and coworkers had found that 10-80μmol/L of galangin inhibited the growth, migration, and invasion of the glioblastoma multiforme cells (U87, U251 and U87-luciferase). Galangin exhibited anti-cancer activity to GBM cells by promoting Skp2 (S-phase-kinase-associated protein 2) degradation (Xiong et al., 2020). In another study, galangin was found to inhibit the growth, migration and invasion of human HCC cell line MHCC97H (Zhong et al., 2020). Galangin also could promote cell apoptosis via down-regulating the expression of long non-coding RNAs (lncRNAs) *H19* (Zhong et al., 2020). However, whether galangin could induce ER stress in colorectal cancer cells is still insufficiently studied.

Reactive oxygen species (ROS) are the products of cellular aerobic metabolism, and regarded as strong oxidants to the biological macromolecules in cells. Due to the existence of anti-oxidants, ROS do not cause damage under normal circumstances. However, once the critical oxidant/anti-oxidant balance is broken, ROS will induce oxidative stress. More importantly, if the oxidative stress is not eliminated efficiently, proteins, DNA or RNA, lipids, and other biomolecules in cells will be oxidative damaged, and eventually lead to changes in signal transduction, mitotic arrest, gene recombination, gene mutation, and subsequently cell death. Anti-oxidant properties of natural products flavonoids play an important role in disease prevention. However, some flavonoid substances under specific conditions show toxic and side effects on cells (Halliwell, 2007). Flavonoids also can promote oxidation in cells (Cao et al., 1997). In detail, when the concentration of flavonoids in cells is particularly high, they could exhibit a pro-oxidation. The pro-oxidation of flavonoids can cause cell toxicity and induce cell apoptosis (Ismail and Alam, 2001; Ueda et al., 2002). It is accepted that flavonoids possessing the same structure and stronger anti-oxidant capacity will have stronger pro-oxidation and then cause greater cell damage (Hadi et al., 2007). Generally, some anti-cancer drugs in clinic like adriamycin can produce high ROS level and then kill tumor cells (Trachootham et al., 2009). Thus, the toxic effect of flavonoids on tumor cells has certain positive significance (Trachootham et al., 2006). For example, the sarsasapogenin from *Anemarrhena asphodeloides* could enhance ROS levels in HeLa cells (Shen et al., 2013). If HeLa cells were treated with anti-oxidant N-acetylcysteine (NAC) before the sarsasapogenin treatment, ROS level and ROS-mediated mitochondrial apoptosis would be inhibited (Shen et al., 2013). Isoobtusilactone A, isolated from *Cinnamomum kotoense*, could induce higher ROS level and up-regulate the expression of CHOP and DR5 in Hep G2 cells (Chen et al., 2012); if NAC was used in advance, ROS generation and the expression of the two proteins would be decreased (Chen et al., 2012). It was also found that the bound polyphenols from inner shell of millet bran could inhibit the growth of a human colorectal cancer cell line (i.e. HCT-116 cells), increase ROS level, and induce cell apoptosis via the NF-κB signal pathway (Shi et al., 2015). Galangin could arrest cell cycle at the G_2/M phase and induce cell apoptosis via increasing intracellular ROS generation in oesophageal carcinoma cells as well as suppressing the expression of Wnt3a and β-catenin (Ren et al., 2016), or accelerate ROS production in human gastric cancer cell line MGC-803 and then induce apoptosis through ROS overload and inactivating the JAK2/STAT3 pathway (Liang et al., 2021). In addition, 25-100 μmol/L of galangin could increase ROS production and inhibit cell invasion and migration by suppressing the epithelial mesenchymal transition (EMT) in human renal cell carcinoma Caki-1 and 786-0 cells (Cao et al., 2016). Whether galangin could induce the ROS-mediated apoptosis in HCT-116 cells also needs an investigation.

The present study thus assessed the activities of galangin to HCT-116 cells. In brief, galangin was measured for its ability to inhibit cell growth, alter cell morphology, cause

cell cycle arresting, induce cell apoptosis, and regulate protein expression. After then, we identified whether both ROS- and ER stress-mediated intrinsic and extrinsic apoptotic pathways were involved in the investigated cell apoptosis. The aim of this study was to reveal whether galangin could exert its apoptosis induction to the cells via both ROS- and ER stress-mediated apoptotic pathways.

MATERIAL AND METHODS

Chemicals and reagents

Galangin (>99% purity) was provided by Shanghai Yousi Biotechnology Co. Ltd. (Shanghai, China). Cell counting kit-8 (CCK-8), cell cycle analysis kit, Annexin V-FITC apoptosis detection kit, Hoechst 33258, ROS assay kit, mitochondrial membrane potential assay kit with JC-1, Fura-2 pentakis (acetoxymethyl) ester (Fura-2 Am), radio immunoprecipitation assay (RIPA) lysis buffer and BCA protein assay kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). TRNzol Universal Reagent, TIANScript RT Kit and RealMasterMix (SYBR Green) were the products of Tiangen Biotech, Co. Ltd. (Beijing, China). Other used chemicals were of analytical grade, while the used water was generated from Milli-Q Plus system (Millipore, New York, NY, USA).

Anti-CHOP, anti-DR5, anti-cytochrome C oxidase IV (COX IV), anti-cytochrome C, anti-Bax, anti-BID, anti-cleaved caspase-3/7/8/9 antibodies, and the secondary antibodies were brought from Cell Signaling Technology (Shanghai) Biological Reagents Co. Ltd. (Shanghai, China), while anti-PIG3 antibody was obtained from Santa Cruz Biotechnology (Shanghai) Co. Ltd. (Shanghai, China).

Cell line and cell culture

HCT-116 cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in the McCoy's 5A medium (Sigma-Aldrich, Co. St. Louis, MO, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C in 5% CO_2.

Assay of growth inhibition and morphological observation

The cells were planted onto 96-well plates at 1×10^4 cells per 100 μL per well to allow to attach. After 24 h, the medium was discarded while galangin (20-160 μmol/L) was added to treat the cells for 24-72 h. 5-Fluorouracil (5-Fu, 100 μmol/L) and dimethyl sulphoxide (DMSO, 0.1%) were used to treat the cells to obtain the respective positive and negative controls, as previously described (Wang et al, 2017). After the treatment, the cells were washed with a phosphate buffer saline (PBS, 0.01 μmol/L, pH 7.0) twice and 10 μL of CCK-8 solution (10 μL CCK-8 in 90 μL PBS of 0.01 μmol/L) was added into each well. The cells were incubated at 37°C for 4 h and then measured for optical density using a microplate reader (Bio Rad Laboratories, Hercules, CA, USA) at 570 nm to calculate growth inhibition. The vehicle-treated cells were taken as 100% viable without any growth inhibition.

The cells (1×10^4 cells per 100 μL per well) were seeded onto 6-well plates. After attachment, 0.1% DMSO or 80-160 μmol/L of galangin were used to treat the cells for 24 h. Then the cells were washed and fixed with 0.5 mL of paraformaldehyde (4%) in the PBS at 4°C overnight, rinsed with PBS twice, and stained with Hoechst 33258 of 0.5 mL for 5 min in the dark at room temperature and then washed with PBS twice. A fluorescence microscope (Olympus, Tokyo, Japan) was used to observe cell morphology with respective excitation and emission wavelengths of 350 and 460 nm.

Assay of cell cycle arrest and flow cytometry analysis

The cells were planted onto 6-well plates for attachment and then treated with 0.1% DMSO or 80-160 μmol/L of galangin for 24 h, washed with PBS, fixed with 70% ice-cold ethanol overnight at 4°C, washed with ice-cold PBS, and then added with 25 μL of propidium iodide (PI, 50 μg/mL) and 10 μL of RNase (100 μg/mL) for 30 min at 37°C in the dark. BD FACSort flow cytometry (Becton Dickson Immunocytometry-Systems, San Jose, CA, USA) was used to analyze cell cycle progression. The portions of the cells in different cell stages (i.e. G_0/G_1, S, and G_2/M phases) were sorted by CellQuest software (ModFit software, Verity Software House, Inc., Topsham, ME, USA).

After treatment with 0.1% DMSO or 80-160 μmol/L of galangin for 24 h, the cells were washed with ice-cold PBS twice and stained with 5 μL of the Annexin V-FITC and 10 μL of PI (20 μg/mL) at 20°C for 20 min in the dark. The cells were analyzed by fluorescent-activated cell sorting using the mentioned flow cytometer and CellQuest software, and designed as necrotic (Q1), early apoptotic (Q2), viable (Q3), and late apoptotic (Q4) cells.

Measurement of mitochondrial membrane potential, ROS, and Ca^{2+}

The cells were seeded onto 6-well plates and treated with 0.1% DMSO or 80-160 μmol/L of galangin for 24 h. Then the cells were re-suspended with fresh medium and 1 mL of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolocarbocyanine iodide) was added for 20 min at 37°C. The cells were washed twice with Dulbecco phosphate-buffered saline (DPBS), re-suspended with 2 mL of fresh medium and then subjected to fluorescence-activated cell sorter (FACS) analysis to evaluate the loss of

mitochondrial membrane potential (MMP) (Salvioli et al., 1997). The used excitation and emission wavelengths were 485 and 590 nm, respectively.

The cells were treated as above, washed with PBS, stained with 1 mL of DCF-DA (2',7'-dichlorofluorescein, 10 μmol/L), re-incubated for 20 min at 37°C, and washed with fresh medium three times. DCF-DA is a cell-permeant dye. Fluorescence intensity was detected by a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) at 488/525 nm with a 525 nm cutoff to report relative ROS level as previously described (Boissy et al., 1989).

To evaluate intracellular Ca^{2+} ($[Ca^{2+}]_i$), the cells were treated as above, rinsed with Krebs-Ringer buffer (pH 7.4) including 37 mmol/L of NaCl, 5 mmol/L of KCl, 1 mmol/L of $MgCl_2$, 1.5 mmol/L of $CaCl_2$, 10 mmol/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 25 mmol/L of D-glucose. Then the cells were applied with Fura-2 AM (5 μells) and re-incubated for 1 h at 37°C, washed twice, and re-suspended in the Krebs-Ringer buffer. A fluorescence spectrometer was used to measure the fluorescence (F_s) at 510 nm emission wavelengths and 340−380 nm excitation wavelengths, respectively. Triton X-100 (0.1%, v/v) treated cells were used to measure maximal fluorescence (F_{max}) and then 10 mmol/L of EGTA (ethylene glycol tetra-acetic acid, pH 9.0) was added immediately to determine minimal fluorescence (F_{min}). An equation $[Ca^{2+}]_i = k_d (F_s - F_{min})/(F_{max} - F_s)$ was used to calculate intracellular Ca^{2+} ($[Ca^{2+}]_i$). K_d is a dissociation constant (224 μmol/L) of Fura-2 and Ca^{2+}. The control cells were set with relative intracellular Ca^{2+} level of 100%.

RNA isolation and quantitative real-time PCR

The cells (5-10×10^6 cells per chamber) were seeded onto 5 cm culture dishes and applied with 0.1% DMSO or 80-160 μmol/L of galangin for 24 h. Total RNA was isolated with TRNzol Universal Reagent (Tiangen Biotech, Co. Ltd, Beijing, China), and complementary DNA (cDNA) was synthesized with reverse transcriptase and oligo$(dt)_{18}$ primer using the TIANScript RT Kit according to the manufacturer's protocol. A 7500 Real-Time PCR System (Applied Biosystems, CA, USA) was used to perform qRT-PCR and thermo-cycling ampification were as following: 95°C initial activation for 60 s, followed by 40 cycles of denaturation 95°C for 15 s; 60°C annealing for 20 s and 68°C extension for 32 s. Relative expression levels of the target genes were determined using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001). The β-actin housekeeping gene was used as an internal control. The used primers synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) had the sequences given in Table 1.

Western blot analysis

The cells were planted onto 5 cm culture dishes overnight, treated with 0.1% DMSO or 80-160 μmol/L of galangin at 37°C for 24 h, rinsed with PBS, and lysed with 0.5 mL of RIPA lysis buffer for 30 min on ice. BCA Protein Assay Kit was used to quantify protein contents. The protein samples (50 μg) were separated by 12 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electro-transferred onto nitrocellulose membranes, blocked with 5% fat free milk at 37°C for 60 min and incubated in the primary antibodies at 4°C overnight. The membranes were washed three times with TBST buffer (containing 10 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20, pH 7.6,), stained with the secondary antibodies at 37°C for 60 min. The antibody-bound proteins were detected by ImageQuant LAS 500 (Fujifilm, Tokyo, Japan).

Statistical analysis

All values are expressed as mean values or mean values ± standard derivations from three independent experiments and analyses. Statistical significance between different groups was analyzed by one-way analysis of variance (ANOVA) with Duncan's multiple range tests using the SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined at $P < 0.05$.

RESULTS

Growth suppression and morphology changes of the galangin-treated cells

The results demonstrated that galangin at the used doses of 20-160 μmol/L had growth suppression on HCT-116 cells (Fig. 1). For the positive cells with 5-FU treatment alone, the measured values of growth inhibition at 24, 48 and 72 h were 79.1%, 70.8 and 69.5%, respectively. In the galangin-treated cells, higher galangin dose and longer treatment time mostly caused greater growth inhibition on the cells; for example, galangin of 160 μmol/L led to respective growth inhibition of 89.1%, 93.1%, and 95.0% at the treatment times of 24, 48, and 72 h. Based on the obtained data, the IC_{50} values of galangin corresponding to the treatment times of 24, 48, and 72 h were thus estimated to be 97.7, 86.2, and 80.8 μmol/L. The results thus proved that galangin had *in vitro* inhibitory effect on HCT-116 cells in both dose- and time-dependent manners.

HCT-116 cells treated with galangin for 24 h also had morphology changes (Fig. 2). Compared with the control cells, the galangin-treated cells showed typical apoptotic morphology, for example, concentration of cytoplasm, reduction of cell volume, condensed chromosome and formation of apoptotic bodies. This fact implied that

Table 1: The primers used in the PCR analyses of this study

Genes	Sense	Antisense
β-Actin	5'-AACACCCCAGCCATGTACG-3'	5'-ATGTCACGCACGATTTCCC-3'
CHOP	5'-GCCAATGATGTGACCCTCAAT-3'	5'-CCTGGAAATGAAGAGGAAGAA-3'
DR5	5'-AAGACCCTTGTGCTCGTTGT-3'	5'-GACACATTCGATGTCACTCCA-3'
Bax	5'-AAGCTGAGCGAGTGTCTCAAG-3'	5'-CAAAGTAGAAAAGGGCGACAAC-3'
AIF	5'-GAATTCGCAATCCGTTGGAGTC-3'	5'-GGATCCCCACGACCACTTTGTC-3'
PIG3	5'-CGCTGAAATTCACCAAAGGTG-3'	5'-AACCCATCGACCATCAAGAGC-3'

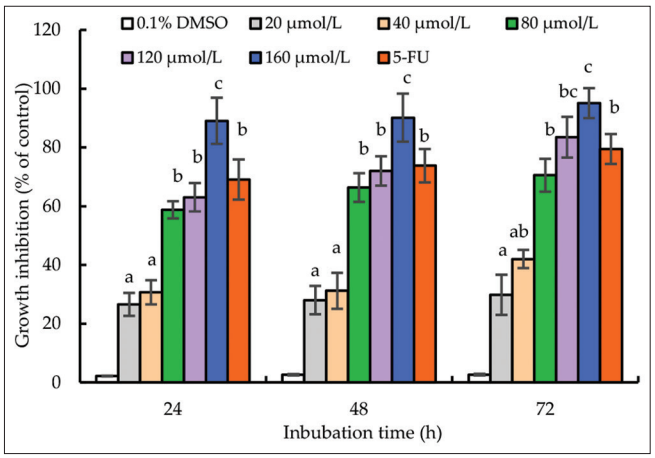

Fig 1. The anti-proliferation of galangin of 20-160 (μmol/L) towards HCT-116 cells with treatment times of 24, 48, and 72 h, respectively. Different lowercase letters above the columns of same group indicates that the mean values differ significantly ($P < 0.05$).

galangin could injury the cells while the cells after galangin exposure were in the process of apoptosis.

Cell cycle arresting and apoptosis of the galangin-treated cells

The cell cycle is divided into two stages: interphase and division. The G_0/G_1 phase is called synthesis phase. The S phase is the stage of DNA synthesis, while the G_2/M phase refers to the late stage of DNA synthesis. The detected cell cycle progression of HCT-116 cells with various treatments was thus analyzed using the flow cytometry (Fig. 3). In the control cells, respective portions of S, G_0/G_1 and G_2/M phases were 72.5%, 15.8% and 11.7% (Table 2). However, cell treatment with 80-160 μmol/L of galangin caused a decrease in cell number in both S and G_2/M phases, and dose-dependently led to increased cell number in the G_0/G_1 phase. For example, respective cell portions of the S, G_0/G_1 and G_2/M phases after galangin exposure of 160 μmol/L were 36.6%, 60.0% and 3.4% (Table 2). The results thus suggested that galangin had an ability to interfere with cell cycle distribution of HCT-116 cells by arresting the cell cycle progression at the G_0/G_1 phase.

Furthermore, a date comparison of the flow cytometry analysis results also suggested that galangin had apoptosis induction to the cells (Fig. 4 and Table 3). It was found

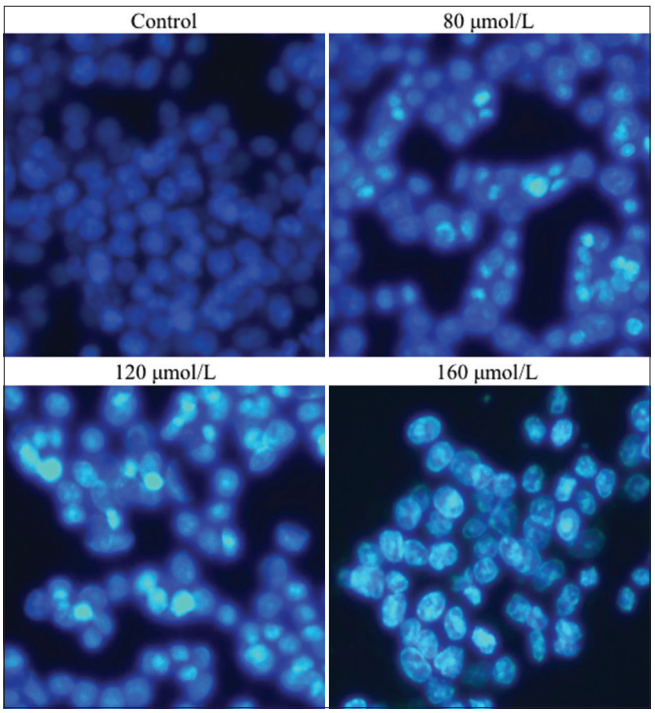

Fig 2. Morphological changes of HCT-116 cells exposed to 80-160 μmol/L of galangin. Apoptotic cells show concentrated cytoplasm, agglutinated chromatin, incomplete and swollen mitochondrial membrane.

that the control cells were detected with 95.8% viable cells and 1.7% apoptotic cells (early plus late apoptotic cells), whereas the cells treated with 80-160 μmol/L of galangin showed decreased viable cells but increased apoptotic cells dose-dependently. For example, 160 μmol/L of galangin was able to cause 41.9% viable cells and 35.9% apoptotic cells. How galangin induced cell apoptosis thus needed a serial expression assay for these genes and proteins to identify the involved pathways.

Intracellular ROS or Ca^{2+} levels and MMP loss of the galangin-treated cells

When HCT-116 cells were treated with galangin at the three doses for 24 h, their intracellular ROS levels were significantly increased (Fig. 5). Compared with the control cells, the cells exposed to 160 μmol/L of galangin received a 3.1-fold increase in ROS generation, whereas those exposed to 80-120 μmol/L also had respective 2.3- and 2.9-fold increase in ROS production. Meanwhile, it was observed

that galangin induced MMP loss in the cells (Fig. 6), because the galangin-treated cells were dose-dependently detected with significant change in MMP (i.e. reduced cell number in Q2). It seemed that overload ROS generation was responsible for MMP alteration and mitochondrial dysfunction. Galangin was thus suggested having an ability to enhance ROS level in the cells and then decrease MMP.

Under normal conditions, the mitochondria uptake and release Ca^{2+} through specific pathway. However, in the high Ca^{2+} loads or oxidative stress situations, Ca^{2+} cycling becomes excessive while MMP decreases dramatically. This brings about failure to maintain Ca^{2+} in the mitochondria, and finally cytosolic Ca^{2+} concentration increases. The present results also showed that galangin could dose-dependently trigger a significant increase in calcium influx across the cell membrane (Fig. 7). When being exposed to galangin of 80-160 μmol/L, the treated cells had respective intracellular Ca^{2+} levels of 121.3%, 145.6%, and 168.3%, which were higher than the intracellular Ca^{2+} level (i.e. 100%) of the control cells.

Mitochondrial stress is generally considered to be accompanied by ER stress signaling. Overall, the galangin-treated cells showed promoted ROS generation together with MMP corruption and Ca^{2+} release, indicating that galangin could stimulate the cells to produce overload ROS products and then induce ER stress in the cells, which broke down MMP and released much Ca^{2+} into the cytosol.

Table 2: Distribution proportions of the S, G_0/G_1, and G_2/M phases in HCT-116 cells with or without 80-160 μmol/L galangin treatment of 24 h

Cell group	S phase (%)	G_0/G_1 phase (%)	G_2/M phase (%)
Control	72.5	15.8	11.7
80 μmol/L galangin	69.1	25.6	5.3
120 μmol/L galangin	49.5	40.9	9.6
160 μmol/L galangin	36.6	60.0	3.4

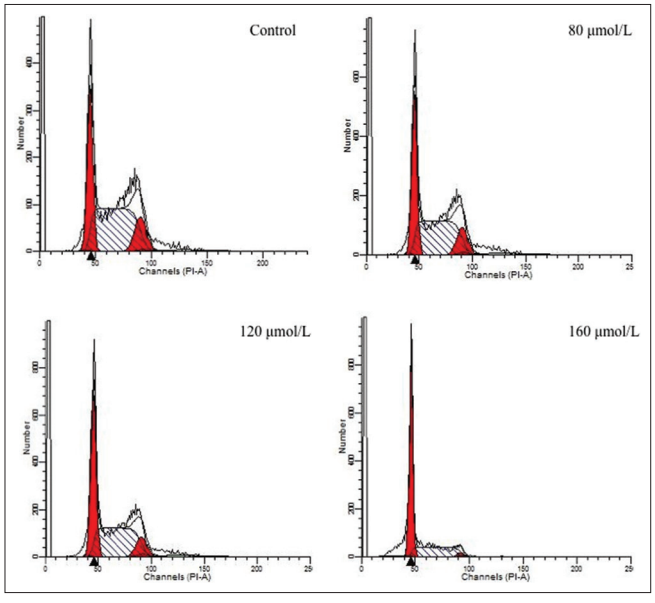

Fig 3. Cell cycle progression of HCT-116 cells treated with galangin at 80-160 μmol/L.

Gene expression changes of the galangin-treated cells

To understand the molecular basis through which galangin induced apoptosis in the HCT-116 cells, the expression levels of mRNAs coding for factors involved in both mitochondrial apoptotic signaling pathway and ER stress were thereby investigated. The results demonstrated that galangin of 80-160 μmol/L was able to up-regulate mRNA expression of these genes namely CHOP (1.9-, and 2.7-folds), DR5 (2.1 and 2.9-folds), Bax (1.9 and 2.5-folds), AIF (1.7 and 2.2-folds) and PIG3 (1.1 and 1.9-folds) in the treated cells (Fig. 8).

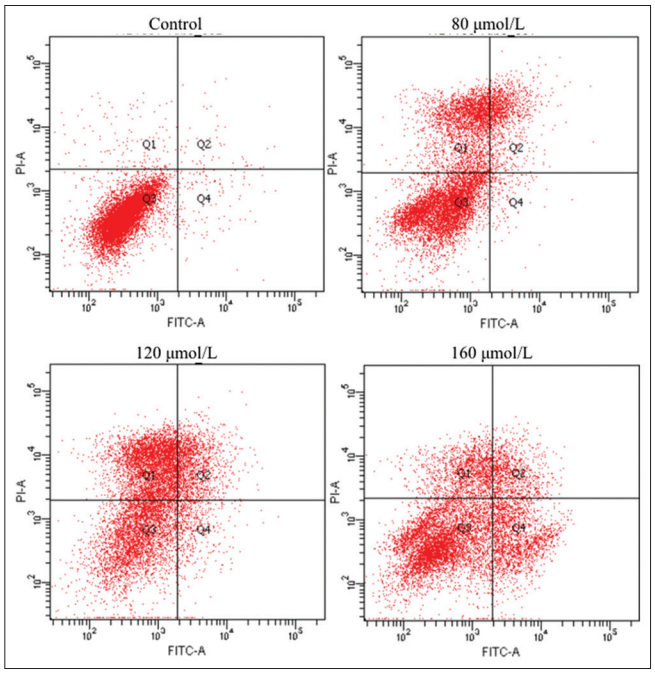

Fig 4. Cell distribution of HCT-116 cells treated with 0.1% DMSO and 80-160 μmol/L galangin for 24 h.

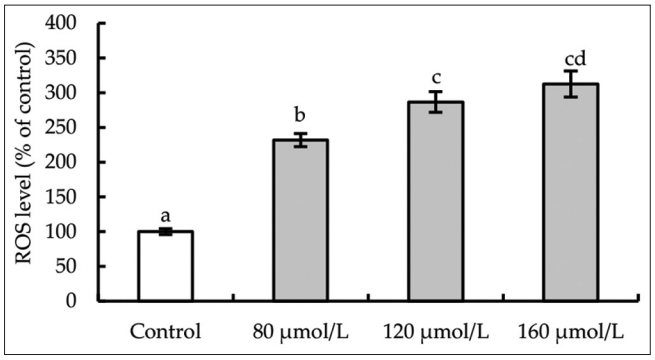

Fig 5. Relative ROS levels of HCT-116 cells treated with galangin at 80-160 μmol/L for 24 h. Different lowercase letters above the columns indicates that the mean values differ significantly ($P < 0.05$).

Protein expression changes of the galangin-treated cells

In consistence with the assaying results of mRNAs expression, the results from western-blot analysis showed that galangin treatment (80-160 μmol/L) could enhance the protein expression levels of CHOP, DR5, cleaved BID, cleaved caspase-3/7/8/9, cleaved PARP and PIG3, Bax, AIF and cytochrome C in the cytosol, but was observed to decrease the protein expression levels of BID (Fig. 9). That is, galangin in the cells could promote ROS formation and induce ER stress, and then activated cleaved caspase-8 by up regulating the expression of CHOP and DR5, cleaved BID into tBid, activated Bax, and promoted the release of AIF and Cyt C into the cytoplasm. AIF released into the cytoplasm could cause directly cell apoptosis, while Cyt C released the cytoplasm might activate cleaved caspases-9/3 and cleaved PARP to cause apoptosis. Galangin could induce ER stress of the cells to activate cleaved caspases-9/7/3, and cleaved ARP, and then led to cell apoptosis. Alternatively, after activating cleaved caspase-8, cleaved caspase-3 and cleaved PARP were also directly activated to promote cell apoptosis. Galangin also regulated the expression of PIG3, which triggered release of AIF from mitochondria and then induced apoptosis. Overall, it was confirmed by these western-blot results that galangin could induce the ROS- and ER stress-mediated intrinsic and extrinsic apoptosis in HCT-116 cells simultaneously.

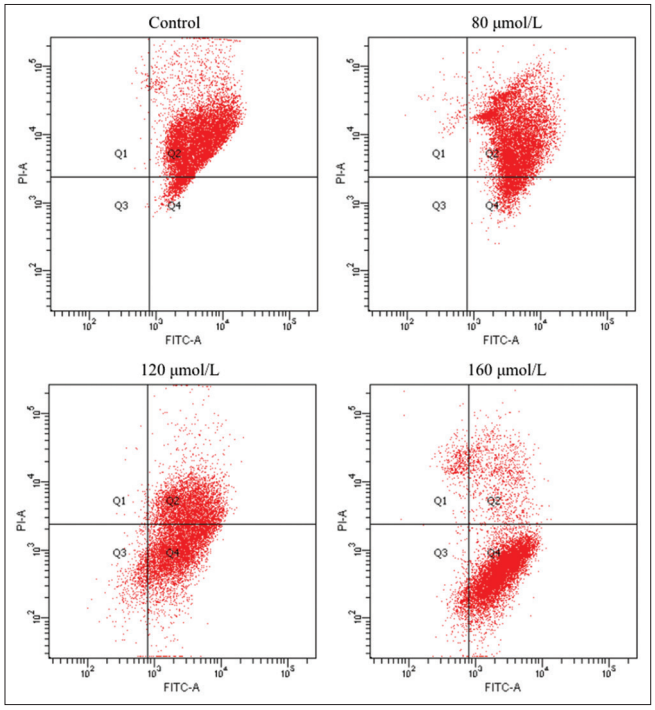

Fig 6. Effects of galangin treatment (80-160 μmol/L) of 24 h on mitochondrial membrane potential loss of the HCT-116 cells at 24 h.

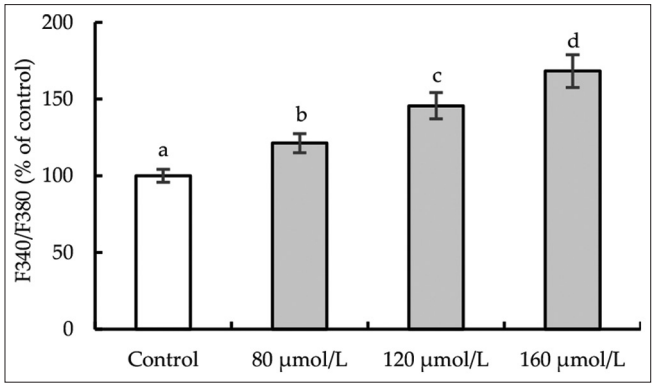

Fig 7. Effects of galangin treatment (80-160 μmol/L) of 24 h on the intracellular Ca^{2+} of the HCT-116 cells at 24 h. Different lowercase letters above the columns indicate significant data differences between different groups ($P < 0.05$).

DISCUSSION

For the past decades, the mechanisms underlying the oncogenesis and metastasis of several cancers including ovarian, lung, colorectal, breast, liver and other cancers have been reveled while anti-carcinogenic compounds from natural plants have been paid special attention, due to their anti-tumor activity and less side effects (Chien et al., 2015; Ziaja-Sołtys et al., 2021; Ullah et al., 2023). Galangin possesses the highest flavonoid level amongst *Alpinia officinarum*, has anti-proliferation, anti-invasion, and is able to cause cell cycle arrest, change morphology and induce apoptosis via different signaling pathways in caner cell lines (Chien et al., 2015). In this study, galangin performed anti-proliferation activity towards the HCT-116 cells in a dose-time dependent manner dose- and time-dependent manners, and also was observed to arrest cell cycle at the G_0/G_1 phase, cause MMP corruption, promote ROS production, enhance Ca^{2+} in the cytosol, and induce cell apoptosis. These results thus provided scientific evidence to support the anti-cancer activity of galangin to cancer cells once more.

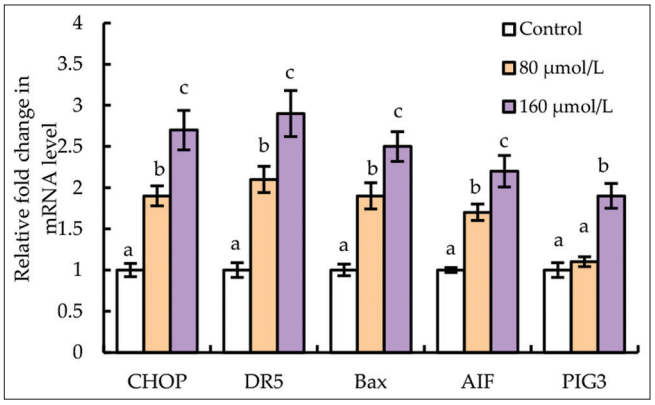

Fig 8. Effects of galangin treatment (80-160 μmol/L) of 24 h on the alteration of gene expression in the HCT-116 cells at 24 h. Different lowercase letters above the columns of same group indicates that the mean values differ significantly ($P < 0.05$).

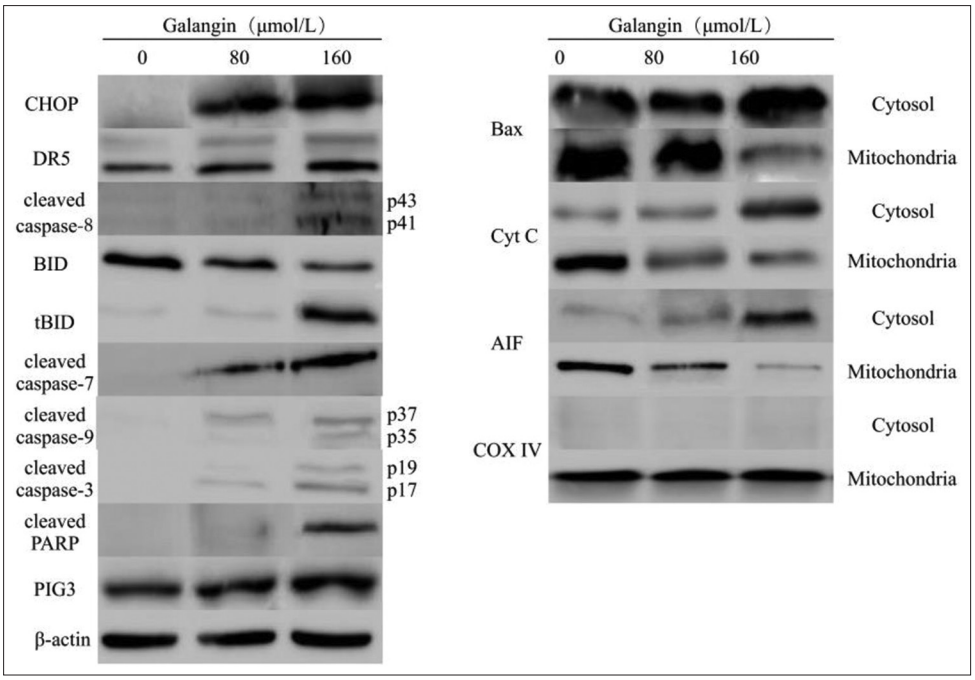

Fig 9. Western blotting assaying results for the expression levels of the apoptosis-related proteins in the HCT-116 cells exposed to galangin at 80-160 μmol/L.

ROS are highly active chemicals in the body. Low ROS level is associated with promotion of mitosis and proliferation, while intermediate ROS level causes cells cycle arresting but high ROS level results in severe oxidative stress, which leads to cell death (either apoptosis or necrosis) (Martindale and Holbrook, 2002). Generally, flavonoids are regarded as one class of the natural antioxidants with anti-oxidant properties. However, in tumor cells, the role of flavonoids is similar to that of oxidants rather than antioxidants, which can increase ROS level in cells and finally promote apoptosis (Romagnolo and Selmin, 2012). Induced apoptosis is accompanied with mitochondrial dysfunction, resulting in MMP decrease. The main reason for MMP decrease is the opening of mitochondrial membrane permeability transport pore (MPTP). Many factors can affect the opening and closing of MPTP (Marchetti et al., 1997). In a study of Marchetti and coauthors, it was found that when cells undergo early apoptosis, the content of anti-oxidants in cells decreased but the ROS levels climbed dramatically (Marchetti et al, 1997). ROS promote and accelerate MPTP opening in the process of apoptosis, while MPTP opening will stimulate cells to produce more ROS. This positive feedback mechanism irreversibly leads to MMP decrease and the cells eventually undergo apoptosis (Marchetti et al, 1997). It has been proved that ROS level was correlated positively with the increase of Ca^{2+} level (Feissner et al, 2011). Thus, in the early stage of apoptosis, after the cells were stimulated by outside signals, ER stress or mitochondrial dysfunction occurred, resulting in the release of Ca^{2+} as well as a large number of ROS. Meanwhile, when ROS level raised to a certain extent, it would further aggravate the release of Ca^{2+} (Feissner et al, 2011). It was thus believed that galangin had capacity to induce the ROS- and ER stress-mediated apoptosis in HCT-116 cells.

The p53-inducible gene 3 (PIG3 or TP53I3) is one of the downstream effectors of the tumor suppressor p53 (Polyak et al., 1997). It has known that PIG3 gene plays two important roles in cells; the first one is associated with cellular response to DNA damage, while another one is responsible for ROS generation. It seems that PIG3 alone is not sufficient enough to provoke apoptosis unless cooperates with p53 and a set of simultaneously activated pro-apoptotic genes induced by ROS to inhibit catalase activity and then subsequently put the cells in an oxidative intracellular environment and finally active apoptosis pathway (Li et al., 2013). In eukaryotic cells, aerobic respiration generates energy and other compounds such as ROS. In normal cells, ROS level is relatively low. If DNA damage occurs at low ROS level, p53 induces a cell cycle arresting to allow DNA repair. PIG3 is activated to take part in DNA repair machinery. In cancer cells, ROS levels are higher than normal cells due to cancer cells need faster metabolism to proliferate. When stimulus is applied to cancer cells, if DNA damage is extremely severe, the pro-apoptotic genes are induced by p53 along with constant ROS generation. PIG3 is activated to participate in DNA damage pathway. This feedback loop leads to high expression of pro-apoptotic genes and increased

production of ROS, which the cells could not conquer and finally undergo apoptotic pathway (Li et al., 2013). In this study, possible mechanism of cell apoptosis induced by galangin towards HCT-116 cells was identified. It is observed that the mRNA level of PIG3 gene in the treated cells significantly increased, indicating that oxidative stress triggers PIG3 activation, which then accelerated ROS generation. In such an oxidative environment, oxidative damage of mitochondria could not be repaired while cell apoptosis via mitochondria pathway was then activated. It has been demonstrated that under such oxidative stress, p53 can translocate to mitochondria to breakdown MMP, and the consequence of which is that the permeability transition pores of the outer membrane open while the mitochondrial membrane become permeable (i.e. leading to mitochondrial depolarization) (Karbowski and Youle, 2003). Moreover, p53 forms an inhibitory complex with Bcl-2 and Bcl-xl proteins to dysfunction these anti-apoptotic proteins, and p53 may induce the expression of Bax and direct the release of AIF from the mitochondria to the cytosol (Minna et al., 2014; Tang et al., 2014). It thus was reasonable that galangin exposure of HCT-116 cells could cause PIG3 up-regulation, increased ROS generation, mitochondria potential collapse, release of AIF and Ca^{2+} to the cytosol, and finally cell apoptosis.

ER is widely distributed in cells. It is the largest organelle in cells and maintains important physiological functions. The main tasks of ER are protein synthesis, folding, transport, signal peptide recognition, glycosylation modification, Ca^{2+} storage and release, and intracellular Ca^{2+} redistribution (Schröder and Kaufman, 2005). Ribosomes are the main sites for protein synthesis. However, some proteins are transferred from ribosomes to ER to continue synthesis. Proteins must fold into appropriate conformations to achieve their cellular functions. Unfolded or misfolded proteins are harmful to cells and even cause cell death (Hetz, 2012). When cells suffer from hypoxia, starvation, Ca^{2+} imbalance, free radicals or drugs, the homeostasis of ER is broken, resulting in a series of molecular and biochemical changes like unfolded protein response (UPR), ER overload response (EOR) and sterol regulatory cascade, which are collectively referred to as ER stress. To overcome ER stress, ER organelles have a specific signal pathway called ER stress response pathway, which involves at least four response mechanisms (Galluzzi et al., 2016). The suggested mechanisms include: (1) reduction of new proteins synthesis; (2) up-regulation the expression of ER chaperone genes and proteins to eliminate unfolded proteins; (3) activation of nuclear factor-kappa B (NF-κB); and finally, (4) if ER damage is too serious or the homeostasis does not recover within a certain period of time, apoptosis will occur (Galluzzi et al., 2016). CHOP gene is called growth arrest and DNA damage inducible gene 153 (GADD153) or DNA damage inducible transcription 3 (DDIT3), and is a marker of ER stress (Zinszner et al., 1998). In this study, when galangin was applied in the HCT-116 cells, CHOP expression was up-regulated both at mRNA and protein levels, indicating that galagnin induced ER stress in the cells. Other researchers had found the similar results when using different cancer models (Su et al., 2013; Song et al., 2017).

Both ROS- and ER stress-mediated endogenous and exogenous apoptosis pathways were regarded to be involved in the apoptosis induction of galangin to HCT-116 cells. When HCT-116 cells were exposed to galangin, they obtained an increase in intracellular ROS level that subsequently caused ER stress. Cleaved caspase-9 was activated and so as cleaved caspase-7 and cleaved caspase-3; cell apoptosis was therefore triggered. ER stress caused CHOP over-expression, which up-regulated the expression of DR5 in its downstream. Pro-caspase-8 was self-sheared and activated to cleaved caspase-8, which then cleaved pro-caspase-3. Activated caspase-3 thus cleaved PARP and induced cell apoptosis. Meanwhile, cleaved caspase-8 also could cleave the cytoplasmic BID. BID thus activated fragment tBid to translocate to the mitochondria, leading to Bax up-regulation and MMP corruption. Cyt C was released into the cytoplasm, and both caspase-9 and caspase-3 were activated in turn to cause cell apoptosis. On the other hand, AIF release also induced cell apoptosis. ER stress caused the release of Ca^{2+} from ER, the increase of intracellular Ca^{2+} level, the nonspecific opening of MPTP, the decrease of MMP, the expansion of mitochondria, the enhancement of protein permeability of outer membrane, the release of soluble membrane proteins from the mitochondria, the cascade reaction of cells, the expansion of signal pathways, and finally the apoptosis of cells. All results suggested clearly that galangin in HCT-116 cells could induce both ER stress- and ROS-mediated endogenous and exogenous apoptosis pathways. In consistent with the present results, the assessed substances like sarsasapogenin, xanthatin, trans-anethole, resveratrol, and cabozantinib were also confirmed to cause the ROS- and ER stress-mediated endogenous and exogenous apoptosis pathways in several model cells, including HeLa, pancreatic cancer, liver cancer HepG2, neuroblastoma, and hepatocellular carcinoma cells (Shen et al., 2013; Geng et al., 2023; Harakeh et al., 2023; Jeon et al., 2023; Sun et al., 2023). However, it is also necessary to identify whether other pathways might be involved in the apoptosis induction of galangin to HCT-116 cells.

CONCLUSION

In the present study, a natural oxidant galangin was confirmed to have *in vitro* growth suppression, cell cycle

arresting, and apoptosis induction towards HCT-116 cells, suggesting consistently its anti-cancer effect on the cancer cells. In brief, the results from cell experiments confirmed that galangin could inhibit cell growth in both time-dose-dependent manners, arrest cell cycle progression at G_0/G_1 phase, promote intracellular ROS generation and Ca^{2+} release, decrease MMP, and induce cell apoptosis. Moreover, galangin also could up-regulate the expression levels of CHOP, DR5, cleaved caspases-3/7/8/9, cleaved BID, Bax, AIF and PIG3, and was able to enhance cytochrome C release. It thus concluded that galangin had anti-cancer effect on HCT-116 cells by inducing cell apoptosis via the ROS-mediated intrinsic and ER stress-mediated extrinsic pathways simultaneously.

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Disclosure statement

The authors declare no conflict of interest.

Author's contribution

Bo Wang: Formal analysis, Investigation, Funding acquisition, Methodology, & Writing - Original Draft. Fei-Fei Wu: Formal analysis. Xin Fu: Formal analysis. Li-Li Liu: Methodology. Xin-Huai Zhao: Conceptualization, Supervision, Project administration, & Writing - Review & Editing.

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