Inhibitory effect of hyperoside isolated from *Zanthoxylum bungeanum* leaves on SW620 human colorectal cancer cells via induction of the p53 signaling pathway and apoptosis

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**Abstract.** The present study aimed to demonstrate the antiproliferative effect of hyperoside from *Zanthoxylum bungeanum* leaves (HZL) and explain the underlying molecular mechanisms in the SW620 human colorectal cancer cell line. The cytotoxic effects of HZL were determined using \(\Delta \Psi_m\) -dichlorofluorescin diacetate and 5,5',6,6'-tetrachloro-1',3',3'-tetraethylbenzimidazol-carbocyanine iodide fluorescence spectrophotometry, respectively. Western blot analysis was used to quantify the expression levels of apoptosis-associated proteins. Reverse transcription-quantitative polymerase chain reaction analysis was used to determine the mRNA expression of glutathione peroxidase (GSH-Px) and catalase (CAT). HZL had a marked anti-proliferative effect on the SW620 human colorectal cancer cells by inducing cell cycle G2/M phase arrest and apoptosis, which was associated with an increase in the expression of p53 and p21. Further mechanistic investigations revealed that the induction of apoptosis was associated with increased generation of ROS, reduced \(\Delta \Psi_m\), and upregulation of B-cell lymphoma 2-associated X protein, cytochrome c, caspase-9, apoptotic protease activating factor 1 and caspase-3. The antitumor potency of HZL was also attributed to inhibition of the mRNA expression levels of GSH-Px and CAT. These data indicated that HZL may be involved in the pro-apoptotic signaling of SW620 human colorectal cancer cells via induction of the caspase-dependent apoptosis and p53 signaling pathways.

**Introduction**

Colorectal carcinoma (CRC) is one of the most common types of human malignancy and a leading cause of cancer-associated mortality in developed countries (1). Identifying strategies to fight against CRC is important, as it is an emerging health problem. It is now well documented that phytochemicals, including flavonoids, alkaloids and polysaccharides, are important in anticancer therapy (2,3).

*Zanthoxylum bungeanum*, also known as Sichuan pepper, is a common Chinese culinary herb, growing widely in the Sichuan, Shaanxi, Shandong and Hebei provinces of China. *Z. bungeanum* leaves are generally used as a vegetable (4). Previous studies have reported on *Z. bungeanum* leaves, in which its antioxidant (5-7), antimicrobial (8), antithrombotic (9) and lipid metabolism-regulating effects (10) were evaluated. However, there has been limited focus on the antitumor activity of the components of *Z. bungeanum* leaves. Hyperoside (quercetin-3-O-galactoside; Fig. 1) is one of the primary flavonoid compounds in *Z. bungeanum* leaves. In addition to its potent efficacy for pain relief, anti-inflammatory and antioxidant activity, and its potential for protecting the cardiovascular system (11-13), hyperoside shows antitumor activity in several tumor models, including RL952 endometrial carcinoma cells (14), A549 lung adenocarcinoma cells (15,16), 786-O renal cancer cells (17) and PC3 prostate cancer cells (12). However, the molecular mechanisms underlying hyperoside-induced growth inhibition and apoptosis of CRC cells remain to be fully elucidated.

A number of flavonoids have been reported to exert pro-oxidant actions, which may be an important mechanism underlying their anticancer and apoptosis-inducing properties (18). It is well documented that flavonoids generate reactive
oxygen species (ROS) in cancer cells. Enhanced ROS production leads to the disruption of cellular antioxidant defense systems and the release of cytochrome c from the mitochondria to the cytosol, resulting in apoptotic cell death (19). Tumor cells with higher levels of ROS are more susceptible to cell death, compared with normal cells with lower levels of ROS. Therefore, novel anticancer drugs may have high potential in promoting the production of ROS. Accumulating evidence suggests that the tumor suppressor, p53, is central to the process of ROS-mediated apoptosis (20). In the present study, hyperoside was isolated from Z. bungeanum leaves (HZL) and its effect on caspase-dependent apoptosis and p53 signaling pathways in human SW620 CRC cells was investigated. The results demonstrated that hyperoside induced cell cycle G2/M phase arrest and apoptosis, which was associated with an increase in the levels of p53, p21, B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), cytochrome c, caspase-9, caspase-3 and apoptotic protease activating factor 1 (Apaf-1). The antitumor potency of HZL was also attributed to increasing ROS levels, a reduction in mitochondrial membrane potential ($\Delta \Psi_m$), and downregulation of the mRNA expression of glutathione peroxidase (GSH-Px) and catalase (CAT).

Materials and methods

Materials. The leaves of Z. bungeanum were harvested in Shaanxi, China in August 2015 and were identified according to the identification standards of the Pharmacopoeia of the People’s Republic of China (21). Dulbecco’s modified Eagle’s medium (DMEM), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2’,7’-dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Fetal bovine serum (FBS) was purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 5,5,6,6’-tetrachloro-1,1’,3,3’-tetracyanobenzimidazolyl carbocyanine iodide (JC-I) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The primers for GSH-Px and CAT were designed and synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). Reagents, including enzymes, cofactors and nucleotides for internal standard construction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, were obtained from Takara Biotechnology Co., Ltd.

Preparation of HZL. Z. bungeanum leaves (500 g) were soaked in 70% ethanol (1:10, w/v) for 2.5 h and were sonicated in an ultrasonic bath at 200 kHz at 55°C for 45 min. The samples were filtered, concentrated and then dried using a rotary evaporator. The dried crude extract was added to distilled water and defatted with petroleum ether. The residue was diluted in H2O and extracted using ethyl acetate in triplicate. The resulting fractions were chromatographed over acetonitrile-0.2% acetic acid (15:85, v/v). The chemical identity of hyperoside was confirmed using reverse phase high performance liquid chromatography by comparison with authentic hyperoside (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China).

The hyperoside was dissolved in DMSO immediately prior to use, and the final concentration of DMSO was <0.1% (v/v) in all experiments. The concentrations of hyperoside ranged between 12.5 and 50 µM. DMSO at 0.1% was used as a control. All determinations were performed in triplicate.

Cell culture and treatment. SW620 cells from the American Type Culture Collection (ATCC; Manassas, VA, USA) were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, Inc.) in a humidified 5% CO2 incubator at 37°C. The medium was replaced every 2 days. The SW620 cells were cultured in 24- or 96-well plates at a density of 1-2x10⁴ cells/ml and were grown to ~70% confluence.

Cell viability. The cell survival rate was quantified using a colorimetric MTT assay. MTT was prepared at 2.5 mg/ml in phosphate-buffered saline (PBS). Briefly, 20 µl aliquots of the MTT stock solution were pipetted into each well, and the plates were incubated at 37°C in a humidified 5% CO2 incubator. After 4 h, the medium was removed and DMSO (200 µl) was added to each well to dissolve the formazan. The optical density of each well was measured 10 min later at 570 nm using a spectrophotometer (Tecan Infinite M200 Pro; Tecan Group Ltd., Männedorf, Switzerland).

Flow cytometric analysis for cell cycle phase determination. Cell suspensions (0.5-1x10⁴/ml) were prepared by trypsinization and washed twice with PBS, followed by centrifugation (200 x g) for 5 min at 4°C. The cells were fixed with 70% ethanol at 4°C and resuspended in PBS. After 20 min in the dark and then analyzed using a FACS flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometric analysis of apoptosis. The prepared SW620 cells (1x10⁴/ml) were washed twice with ice-cold PBS and gently resuspended in 500 µl of binding buffer. Subsequently, the cells were stained with 5 µl Annexin V-FITC and agitated well. Finally, 5 µl PI was added to the cells, incubated for 20 min in the dark and then analyzed using a FACS flow cytometer (BD Biosciences).

Western blot analysis. Cell lysates were prepared from confluent 6-well plates (Nalge Nunc International, Penfield,
NY, USA) by scraping into lysis buffer (0.05 M Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS). The total protein content of cell lysates was determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell lysates (30 µg of total protein) were analyzed using 8-12% gradient SDS-PAGE. The proteins were transferred onto an immunoblot PVDF membrane (EMD Millipore, Billerica, MA, USA), which was blocked with 5% skimmed milk in TBS containing 0.1% Tween-20 (TBS-T) at room temperature for 1 h, and incubated overnight at 4°C with the following primary antibodies: Rabbit anti-p53 (4667; 1:1,000), anti-p21 (2947; 1:2,000), anti-Bax (2772s; 1:1,000), anti-cytochrome c (4280s; 1:1,000), anti-Apaf-1 (8969s; 1:1,000), anti-caspase-9 (9508; 1:1,000), or anti-caspase-3 (9662s; 1:1,000) (all from Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were then washed with TBS-T buffer, incubated with the horseradish peroxidase (HRP)-conjugated anti-mouse IgG (14709; 1:1,000; Cell Signaling Technology, Inc.) for 1 h at room temperature, and enhanced using a chemiluminescence ECL assay kit (Amersham; GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturer's protocol. Images of chemiluminescence were captured using a Fujifilm LAS-3000 system (Fujifilm, Tokyo, Japan). The basal levels of proteins were normalized to the protein level of β-actin.

Measurement of ΔΨm. The ΔΨm of the cells was analyzed using fluorescence spectrophotometry using JC-1 dye. Briefly, the SW620 cells were stained with 5 mM JC-1 at 37°C for 20 min in a 5% CO2 atmosphere. The cells were pelleted by centrifugation (1,000 × g for 5 min at 4°C) and resuspended in PBS. The JC-1 fluorescence of the cell suspensions and PBS controls were measured in triplicate in Costar 96-well plates using a microplate reader (Tecan Infinite M200 Pro; Tecan Group Ltd.). Ex/Em (green)/Em (red) = 485/538/590 nm; FL2/FL1 ratios were calculated based on the fluorescence data. Each well was scanned by measuring the intensity of each 25-square grid (1 mm² area), which was arranged in a 5x5 rectangular array (bottom scanning). A higher red-to-green ratio indicated a more polarized or more negative and hyperpolarized mitochondrial inner membrane.

Measurement of intracellular ROS levels. The cellular ROS levels were measured using a dichlorofluorescein assay. The cells were collected and incubated with 100 µM DCF-DA (dissolved in DMSO) for 30 min at 37°C. Subsequently, the cells were washed three times with PBS (pH 7.4) and the relative levels of fluorescence were quantified in a spectrofluorimeter (Tecan Infinite M200 Pro; Tecan Group, Ltd.). The measured fluorescence values were expressed as a percentage of the fluorescence in the control cells.

RT-qPCR analysis. The cells were washed with ice-cold PBS, and RNA was extracted using TRIzol reagent (Biotek Corporation, Beijing, China) according to the manufacturer's protocol. cDNA was synthesized from mRNA using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.), followed by qPCR using a SYBR Premix Ex Taq™ reagent kit (Takara Biotechnology Co., Ltd.) and the ABI Prism 7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reactions were performed under the following cycling conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The housekeeping gene β-actin was used for normalization. All experiments were repeated at least three times. The relative changes in gene expression were analyzed using the 2-ΔΔCq method (22). The sequences for the RT-qPCR primers were as follows: GSH-Px forward, 5'-CCTCTAACAACCTACGGGG AGGAA-3' and reverse, 5'-GGGAAACTCGCTTGTGTCT-3'; CAT forward, 5'-TCCAAGGCIAAGGTATTTGAGC-3' and reverse, 5'-CAACGAGATCCAGTTTACACCTTC-3'; α-actin forward, 5'-CATCCGTAAAGACCTCTATGCGAA C-3' and reverse, 5'-ATGGAGACCCCGATCCCA-3'.

Statistical analysis. All data are expressed as the mean ± standard deviation of at least three independent determinations for each experiment. Statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance with Duncan's multiple range test was used to examine differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of HZL on SW620 cell growth inhibition. The growth inhibiting effect of HZL was assessed using an MTT assay and microscopic analysis, as demonstrated in Fig. 2A. Time- and concentration-dependent growth inhibition was observed in the SW620 human CRC cell line. Following treatment with HZL for 24, 48 and 96 h, the half maximal inhibitory concentration values were 72.35±5.46, 36.41±1.36 and 19.51±4.95 µM, respectively. The viabilities of cells treated with HZL (12.5, 25 and 50 µM) for 48 h were significantly (P<0.01, P<0.01 and P<0.01, respectively) lower, compared with cells in the control group (Fig. 2A). Additionally, microscopic analysis demonstrated prominent morphological changes resembling cell swelling, irregularities in the plasma membrane and the formation of membrane blebs and vacuoles, to a significantly higher extent, compared with the control cells (Fig. 2B). This provided morphological evidence to qualitatively demonstrate that HZL induced apoptosis, which was in agreement with the results of the MTT assay described above.

The present study then investigated whether the cytotoxicity of HZL to SW620 cells is associated with the induction of cell cycle arrest and apoptosis. Cell cycle arrest and apoptosis were determined using flow cytometry following treatment of the SW620 cells with 12.5, 25 and 50 µM HZL for 48 h. Characteristic examples of the observations are demonstrated in Fig. 2C and D. The percentages of SW620 cell apoptosis were 17.3, 21.2 and 32.9%, in the 12.5, 25 and 50 µM HZL treatment groups, respectively (Fig. 2C). These results demonstrated that HZL induced apoptosis in a dose-dependent manner, which was consistent with the results of the MTT assay. The percentage of G2/M phase cells in the groups treated with 12.5, 25 and 50 µM HZL was significantly elevated, whereas the percentages of G1/G0 and S phase cells were decreased by HZL (Fig. 2D).
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In order to examine the mechanisms underlying HZL-induced cell cycle arrest and apoptosis, activation of the p53-mediated signaling network in SW620 cells was investigated using western blot analysis (Fig. 3A). Compared with the control group, the protein expression levels of p53 and p21 were increased by 116 and 198%, respectively, in the SW620 cells treated with 25 µM HZL for 48 h (Fig. 3B and C). The expression levels of pro-apoptotic proteins Bax, cytochrome c, Apaf-1, caspase-9 and caspase-3 were increased in a dose-dependent manner (Fig. 3D-H). These data suggested that HZL induced apoptosis of the SW620 cells through p53- and caspase-dependent apoptotic pathways.

p53-mediated apoptosis. In order to examine the mechanisms underlying HZL-induced cell cycle arrest and apoptosis, activation of the p53-mediated signaling network in SW620 cells was investigated using western blot analysis (Fig. 3A). Compared with the control group, the protein expression levels of p53 and p21 were increased by 116 and 198%, respectively, in the SW620 cells treated with 25 µM HZL for 48 h (Fig. 3B and C). The expression levels of pro-apoptotic proteins Bax, cytochrome c, Apaf-1, caspase-9 and caspase-3 were increased in a dose-dependent manner (Fig. 3D-H). These data suggested that HZL induced apoptosis of the SW620 cells through p53- and caspase-dependent apoptotic pathways.

ROS-mediated apoptosis. The enhancement of ROS production has long been associated with the apoptotic response induced by anticancer agents. Mitochondria are key organelles for cell survival and area source of ROS generation during apoptosis. In the present study, JC-1 staining was applied to detect changes in the $\Delta \Psi_{m}$ of SW620 cells. The ratios of JC-1 red-to-green fluorescence intensities decreased by 21.8, 41.6 and 43.7% when the SW620 cells were treated with 12.5, 25 and 50 µM HZL for 48 h. These results revealed that HZL induced apoptosis through the mitochondrial pathway (Fig. 4A). The generation of ROS was increased to 139.1% of the untreated control when treated with 25 µM HZL for 48 h (Fig. 4B). This indicated that HZL caused the production of ROS. ROS are created through aerobic metabolism and are rapidly removed by endogenous antioxidants, including superoxide dismutase (SOD), CAT and GSH-Px. The results of the present study demonstrated that the mRNA expression levels of CAT and GSH-Px were decreased following treatment with 25 µM HZL, whereas the ROS levels were increased (Fig. 4C and D).

Discussion

Flavonoids are polyphenolic compounds, which are found ubiquitously in plants. The role of dietary flavonoids in cancer prevention has been widely discussed. Several mechanisms of action of flavonoids have been identified, including carcinogen inactivation, antiproliferative activity, cell cycle arrest, induction of differentiation and apoptosis, inhibition of angiogenesis, antioxidant activity and reversal of multidrug resistance, or a combination of these mechanisms (23). Hyperoside is one of the major active compounds of Z. bungeanum leaves; however, the anticancer activity of hyperoside has not been reported previously. To the best of the authors' knowledge, the present study is the first to investigate the molecular effects of HZL on human SW620 CRC cells.

Inhibition of the cell cycle has become an important target for the management of cancer. In the present study, the data obtained on the cell cycle distribution of the cell population indicated that HZL may have mediated the growth inhibition of SW620 cells via perturbation in the G2/M phase of the cell cycle. It has been demonstrated that flavonoids induce the inhibition of growth via cell cycle arrest in the G2/M phase of several cancer cell lines, including ovarian carcinoma...
(SKOV3) and osteosarcoma (U2OS) (24), anaplastic thyroid carcinoma (25), and several digestive system cancer cell lines (26,27), which in itself is inconsistent with the results obtained in the present study. However, Zhang et al. (28) investigated the effect of hyperoside on human osteosarcoma cells and found that, following treatment with hyperoside, cells were arrested in the G0/G1 phase. Several studies have also indicated that arrest of the cell cycle in the G0/G1 phase is induced by flavonoids in human osteosarcoma MG-63, HeLa, prostate and breast cancer cells (29-31). Therefore, the cellular changes referred to above suggest that the cell cycle arrest caused by flavonoids is cell type- and concentration-dependent.

Tumor suppressor p53 is important in mediating cell responses to various stresses, predominantly by inducing or suppressing a number of genes involved in cell processes, including cell death, cell cycle arrest, apoptosis, senescence and DNA-repair (32,33). p21 is an important checkpoint gene in the cell cycle, and it is also regulated by the transcription of p53; it contributes to the repair of damaged cells by terminating DNA synthesis and inactivating the nuclear antigen in proliferating cells (34). As demonstrated in Fig. 3, the present

Figure 3. Effect of Zanthoxylum bungeanum on p53-associated proteins. SW620 cells were exposed to 12.5, 25 and 50 µM hyperoside for 48 h. (A) Protein expression of p53-associated proteins. Quantification of (B) p53, (C) p21, (D) Bax, (E) Cytc, (F) caspase-9, (G) caspase-3 and (H) Apaf-1. *P<0.05 and **P<0.01 vs. Control. Bax, Bcl-2-associated X protein; Cytc, cytochrome c; Apaf-1, apoptotic protease activating factor 1.
study detected upregulation in the protein expression levels of p53 and p21, suggesting that the upregulation of p53 and p21 was involved in HZL-induced G2/M cell-cycle arrest.

Apoptosis is a regulated programmed cell death process, which provides an effective non-inflammatory mechanism for the removal of redundant or damaged cells from tissues, thereby attaining tissue homeostasis (35). Tumor suppressor p53 is also an important activator of the intrinsic apoptotic pathway. The upregulation of p53 is commonly observed following exposure to DNA-damaging agents, including oxidative stress or UV light (36). Tumor suppressor p53 has been demonstrated to differentially regulate the levels of Bcl-2 and Bax in vitro and in vivo (37). The increased production of Bax following p53 activation is due to p53 being a direct transactivator of the gene expression of Bax (37). The decreased expression of Bax in the present study was concomitant with the decreased expression of p53 and suggested that the apoptosis induced by HZL in SW620 cells may be controlled by hyperoside via the p53 pathway.

The collapse of $\Delta \Psi_m$ is considered to coincide with the opening of mitochondrial permeability transition pores, leading to the release of cytochrome c into the cytosol. In the cytoplasm, cytochrome c combines with caspase-9, Apaf-1, and dATP to form the apoptosome complex, which in turn activates caspases-9, 3 and 7 (38). Caspase-9 and its cofactor, Apaf-1, are essential downstream components of p53 in Myc-induced apoptosis (39). In the present study, HZL upregulated the expression levels of cytochrome c, Apaf-1, caspase-9 and caspase-3, compared with the control group (Fig. 3). These findings suggested that p53 and the caspase-dependent signaling pathway were involved in HZL-induced apoptosis.

Reduced $\Delta \Psi_m$ can lead to increased generation of ROS and apoptosis. In this context, Zeng et al. (40) suggested that hyperoside alters the $\Delta \Psi_m$ and induces mitochondrial collapse. Similar results were obtained in the present study when SW620 cells were treated with HZL. These findings, along with other those of others, suggested that hyperoside acts as an antiproliferative agent through the overproduction of ROS, induction of apoptosis and loss of $\Delta \Psi_m$. The p53 protein has been suggested as a critical regulator of intracellular ROS levels. Upon activation following DNA damage, p53 can activate several genes, which results in increased...
ROS generation, contributing to the induction of apoptosis in cells with unrepaired DNA damage (41).

It has been found that human colorectal tumors (adenomas and carcinomas) have increased levels of different markers of oxidative stress, including increased levels of ROS, nitric oxide (NO), lipid peroxides, GSH-Px and CAT (43). Alternative mechanisms for flavonoid pro-oxidant toxicity involve numerous peroxidases, which catalyze the oxidation of polyphenols. The intracellular phenol radicals (redox-cycling phenols) formed by myeloperoxidase in neutrophils also induce lipid peroxidation and co-oxidize GSH to form thiol radicals with concomitant oxygen release. The levels of CAT may be critical in cell-induced resistance to the effects of anticancer drugs, which upregulate p53 (44). Kang et al (45) demonstrated that p53 upregulates ROS generation by suppressing the activity of CAT in response to DNA damage. p53-mediated GSH depletion was found to enhance the cytotoxicity of NO in silibinin-treated human cervical carcinoma and HeLa cells (46). In the present study, HZL inhibited the mRNA expression levels of CAT and GSH-Px in a dose-dependent manner, suggesting that p53 was associated with the expression of genes encoding antioxidant enzymes.

The complex mechanism of HZL-induced cell cycle arrest and apoptosis in SW620 cells is demonstrated in Fig. 5. The results revealed that HZL increased the accumulation of ROS via inhibition of the gene expression of CAT and GSH, which resulted in cell cycle G2/M phase arrest and apoptosis. Further mechanistic investigations revealed that the induction of cell cycle G2/M phase arrest and apoptosis was associated with the upregulated expression of p53, p21 and Bax in the SW620 cells. Consequently, the ∆Ψm was reduced, which accelerated the release of cytochrome c into the cytoplasm leading to apoptosis of the SW620 cells via the caspase-dependent pathway. These findings suggested that hyperoside may be a potent chemopreventive agent against human CRC, acting through induction of the caspase-dependent apoptosis and p53 signaling pathways. Further investigations are warranted to determine the clinical efficacy and precise molecular mechanism by which hyperoside inhibits cancer cell growth.

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