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Gracillin induces apoptosis in HL60 human leukemic cell line via oxidative stress and cell cycle arrest of G_1

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Gracillin, a kind of steroidal saponin isolated from the root bark of wild yam *Dioscorea nipponica* has been reported to exert antitumor activity. In the present study, we investigated the anticancer activity of gracillin against HL60 cells, and evaluated the possible mechanism involved in its antineoplastic action. The cell proliferation was evaluated by cell counting Kit-8 (CCK-8) assay, gracillin inhibited the growth of HL60 cells in a time- and concentration-dependent manner. Flow cytometry was used to analyze the cell cycle distribution whereas Annexin V-FITC/PI flow cytometry analysis was carried out to confirm apoptosis induced by gracillin, Our results demonstrated that gracillin could induce cell cycle arrest of G_1 and apoptosis in HL60 cells. Furthermore, based on the biochemical methods, induction of oxidative stress by gracillin was indicated by increased the content of malondialdehyde (MDA), and decreased superoxide dismutase (SOD) activity. In addition, real time-PCR verified the expression of apoptosis-related genes, the mRNA level of Bcl-2 was decreased dramatically, while Bax was remarkably increased by gracillin. Taken together, gracillin could induce cell cycle arrest, oxidative stress, and apoptosis in HL60 cells, and has the potential to be developed as an antitumor agent.

1. Introduction

Apoptosis is a physiological process of cellular suicide. Deregulated cell proliferation and inhibition of cell death evoke uncontrolled development of cancer (Kaufmann and Gores 2000). The goal of cancer therapy is to induce selective apoptosis on cancer cells by activating apoptotic signaling pathways, and exert no harmful effects on normal cells (Gerl and Vaux 2005; Wong 2011). At the biochemical level, apoptosis is mediated by the activation of a class of cysteine proteases called caspases. In mammalian cells, caspase activation mainly occurs either through death receptor activation or mitochondrial membrane permeabilization (Danial and Korsmeyer (2004). The mitochondrial pathway of apoptosis is regulated principally by the Bcl-2 protein family (Hasnan et al. 2010; Coultas and Strasser 2003). Oxidative stress has usually been observed during the process of apoptosis in cells subjected to anticancer drug treatment (Yu et al. 2013). Increased ROS levels might lead to DNA damage and these damaged cells subsequently undergo either cell cycle arrest to facilitate DNA repair, or induce apoptosis to eliminate the excessively damaged cells (Tor et al. 2014). The plant kingdom has a long history as a source of anticancer agents, and gives prominent impact on the modern drug development process (Newmann and Cragg 2012).

Gracillin is a steroidal saponin, which is found in a variety of plants including fenugreek (*Trigonella foenum-graecum*), roots of wild yam (*Dioscorea villosa*), *Solanum incanum*, and *Solanum xanthocarpum* (Raju and Mehta (2009). The inhibitory effect of gracillin on tumor cell growth has been known for years, but the molecular mechanism is not yet clear (Srinivasan et al. 2009; Hou et al. 2004; Wei et al. 2013; Hsieh et al. 2013). Therefore, this study intended to explore the inhibition effect of gracillin on human leukemic HL60 cells, as well as its potential mechanism. It will provide a certain level of theoretical and scientific basis for future research.

2. Investigations and results

2.1. Gracillin inhibited proliferation of HL 60 cells

The inhibitory effect of gracillin on the proliferation of HL60 cells was measured by CCK-8 assay. The cells were treated with different concentrations (6.25,12.5, 25, 50, 100 μM) of gracillin for Fig. 1 different periods of time (24, 48 and 72 h). As shown in Fig. 2, gracillin significantly inhibited the growth of HL-60 cells in a dose-and time-dependent manner. The half-inhibitory concentration (IC_{50}) of gracillin on HL60 cells for 24, 48 and 72 h were approximately 98.37 μM, 56.37 μM, 36.8 μM, respectively. Based on the cytotoxic effect, 12.5, 25 and 50 μM of gracillin, and incubation time of 48 h were selected for further analysis.

2.2. Gracillin induced apoptosis in HL60 cells

To determine whether gracillin induces apoptosis in HL60 cells, the Annexin-V and PI double staining was carried out. The addition of both early and late apoptotic cells (Annexin V-FITC positives) was defined as the total percentage value of apoptotic cells. The ratio of the apoptotic cells in HL60 cells significantly

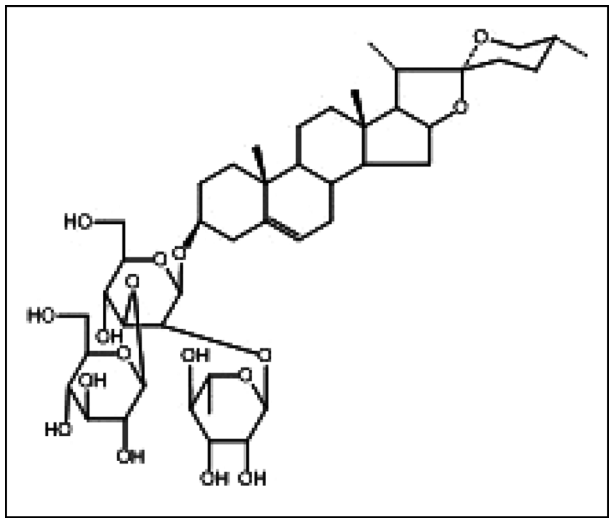

Fig. 1: Structure of gracillin.

increased in a dose-dependent manner. After treatment with 50 μM of gracillin for 48 h, the percentage of apoptotic cells increased from 4.8% to 53.9% (Fig. 3). The results suggested that gracillin strongly induced HL60 cell apoptosis.

2.3. *Gracillin induced cell cycle arrest of G_1 in HL60 cells*

To investigate whether gracillin affects the cell cycle distribution in HL60 cells, the cell cycle of HL60 cells was analyzed by PI staining and flow cytometry analysis. As shown in Fig. 4. Gracillin treatment induced cell cycle arrest of G_1 phase in HL60 cells in a dose-dependent manner, the percentage of cells in G_1 phase were 11.04, 12.59, 15.48, 34.5 and 32.88%, respectively.

2.4. *Gracillin induced oxidative stress in HL60 cells*

In order to know the degree of oxidative stress that occurred as a result of treatment with gracillin, we studied the level of MDA and SOD activity in the cells. As shown in Fig. 5, gracillin treatment resulted in a significant increase in the level of MDA accompanied with a significant decrease in SOD activity with a dose-dependent way. Compared the control group, the level of MDA was increased by 240% whereas the activity of SOD was decreased by 62% in the 50 μM group.

2.5. *Gracillin affected the expression of Bcl-2 and Bax*

Bcl-2 is an antiapoptotic protein, whereas Bax is a proapoptotic protein belonging to the Bcl-2 gene family. Together, the levels of these anti-and proapoptotic proteins determine a cell's fate for survival or cell death (Hasnan et al. 2010; Coultas and Strasser 2003). Quantitative real time-PCR was used to determine the expression of Bcl-2 and Bax in HL60 cells. The results showed that gracillin treatment decreased the expression levels of Bcl-2 while increased the expression levels of Bax, so the Bcl-2/Bax ratio was markedly down-regulated (Fig. 6).

3. Discussion

Natural-derived products, regardless if crude extracts or isolated active compounds, had drawn growing attention as agent in cancer therapy, due to their ability to modulate apoptosis (Zhang et al. 2009; Chen et al. 2012; Ji et al. 2002). Previous studies indicated that saponins exhibit anti-tumorigenic effects *via* multiple anticancer pathways because of the great diversity of their structures (Tang et al. 2013; Balsevich et al. 2012). In the present study, we carried out further investigations to elucidate the antitumor activities of gracillin on HL60 cells and the possible mechanisms involved. Our experimental data showed that gracillin significantly inhibited the proliferation of HL 60 cells at low concentrations, the IC_{50} of gracillin to HL60 cells for 72 h after treatment was 36.8 μM. In addition, Annexin V-FITC/PI assay showed that gracillin induced cell apoptosis in HL60 cells, and the combined early and late apoptotic cells (Annexin V positive) were elevated in a dose-dependent manner. These results indicated that gracillin inhibits the proliferation of HL60 cells *in vitro* by induction of apoptosis.

Deregulation of cell cycle progression and evasion of apoptosis are hallmarks of cancer cells (Senderowicz 2004). The nature of cell proliferation is to realize DNA replication constantly

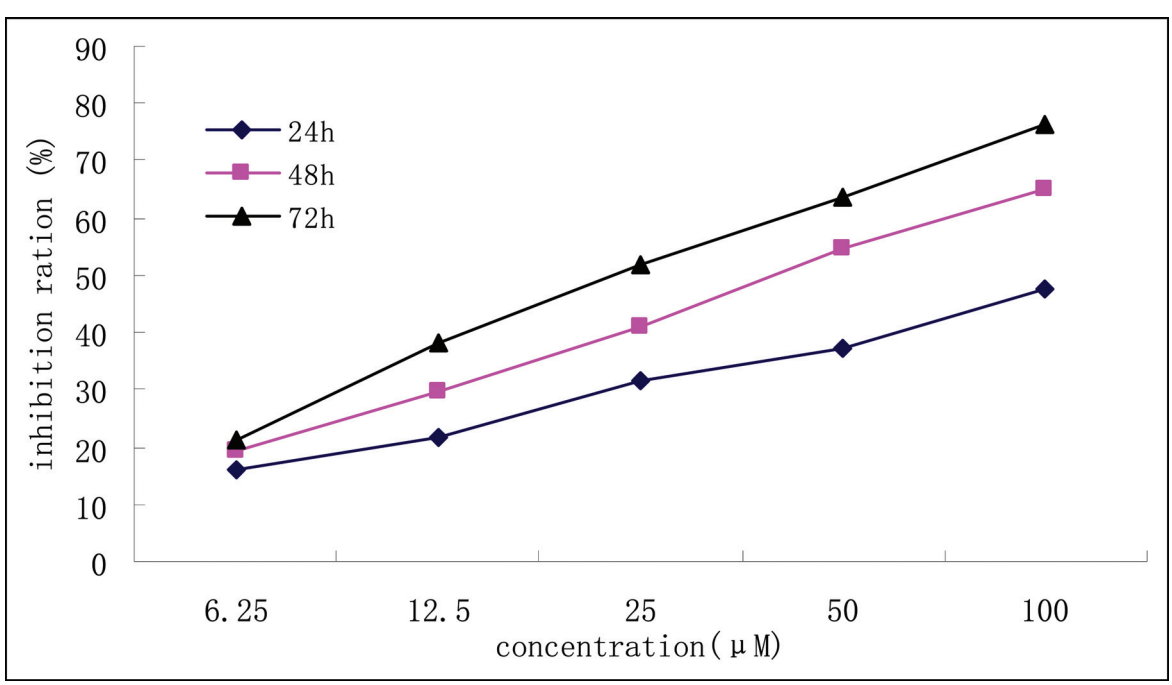

Fig. 2: Inhibitory effect of gracillin on HL60 cells proliferation. Gracillin inhibited the growth of HL-60 cells in a dose-and time-dependent manner.

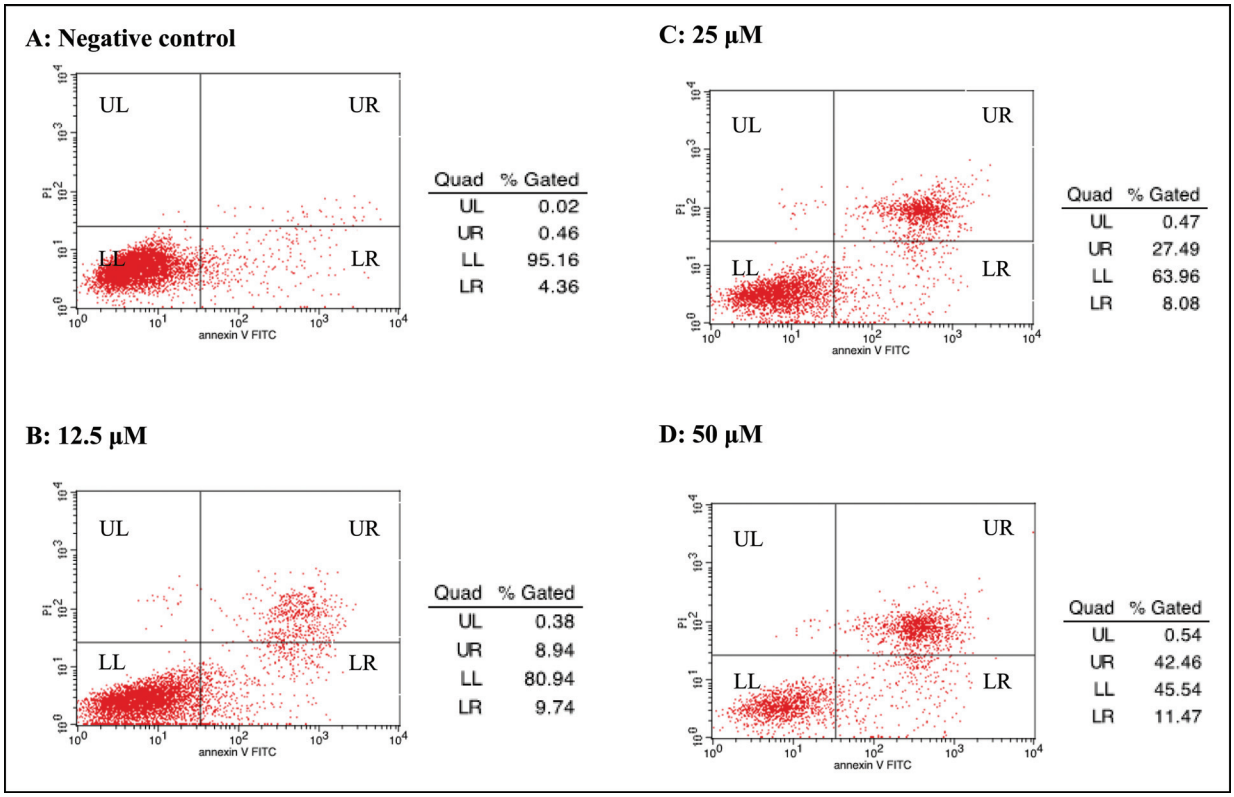

Fig. 3: Effect of gracillin treatment on apoptosis in HL60 cells using Annexin V/PI staining. HL60 cells were treated with various concentrations of gracillin (0, 12.5, 25, 50 µM) for 48 h. Lower left quadrants, viable cells. Upper left quadrants, necrotic cells. Lower right quadrants, early apoptotic cells, Upper right quadrants, late apoptotic cells.

through the cell cycle; when the cell cycle is arrested, the proliferation of tumor cells will be inhibited and the cell apoptotic program is initiated (Wang et al. 2010). Oxidative stress is one of the most important factors involved in formation and growth of tumors (Lecane et al. 2005). The accumulation of intercellular superoxide may lead to the disruption of the mitochondrial membrane potential, release of cyctochrome c into the cytosol, with subsequent activation of the caspase cascade, and apoptosis (Singh et al. 2005).

Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and plays an important role in antioxidant defense in nearly all cells exposed to oxygen (Deng et al. 2012). Malondialdehyde (MDA) is generated from the degradation of polyunsaturated lipids by ROS. It is one of the most frequently used indicators of lipid peroxidation (Gil et al. 2002). In our research, content increase of G_1 phase was observed after treatment of gracillin, and the results demonstrated that gracillin arrested the cell cycle at phase G_1, which prevented cells from progressing towards mitosis. In addition, we found that gracillin treatment significantly increased the concentrations of MDA and decreased SOD activities. On the basis of our data analysis, we have reasons to conclude that gracillin induces apoptosis in HL60 cells is associated with oxidative stress and cell cycle arrest of G_1.

Apoptosis is a gene-regulated event related to special morphological changes such as shrinkage of cell, chromatin condensation and DNA damages (Wyllie 1997). The Bcl-2 family plays a critical role in apoptosis regulation (Adams and Cory 1998). Pro-apoptotic Bax induces the release of cytochrome c and activates the Bax-initiated mitochondrial pathway and the capsese3-dependent apoptotic pathway, whereas anti-apoptotic Bcl-2 blocks mitochondrial apoptosis by blocking the release and oligomerization of Bax. Elevated ratio of Bax/Bcl-2 is an important marker of apoptosis (Youle and Strasser 2008). To investigate the molecular mechanism underlying gracillin-induced apoptosis in HL60 cells, we measured the expression of two key apoptosis-regulated genes Bcl-2 and Bax. The results revealed that gracillin up-regulated the expression of Bax and down-regulated the expression of Bcl-2 in HL60 cells, so the ratio of Bax/Bcl-2 significantly increased. Hence, we suggested that gracillin induced apoptosis in HL60 cells by affecting the expression of bcl-2 and bax.

In conclusion, the present study demonstrated that gracillin significantly inhibited the growth of human leukemic HL60cells at low concentration by inducing apoptosis, oxidative stress and cell cycle arrest. These findings indicate the potential of gracillin to be developed as an antitumor agent. However, further studies are needed to investigate the effects of gracillin in normal cell lines and in animal models, in order to provide a broader experimental basis for clinical application.

4. Experimental

4.1. Chemicals

Gracillin (purity of > 98%) used in the present study was extracted and isolated from *Dioscorea nipponica*.

4.2. Cell line and culture

The human leukemic HL60 cell line was purchased from the KeyGen Serving Science Company (Nanjin, China). HL60 cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heatin-activated fetal bovine serum (FBS; Sijiqing Biological Engineering Materials, Hangzhou, China), and 100 U/ml of both penicillin and streptomycin. Cells were routinely cultured in a humidified incubator (Sanyo XD-101; Sanyo, Osaka, Japan) at 37 °C with 5% carbon dioxide.

4.3. Analysis of cell viability

Cell viability was detected by CCK-8 assay. $5*10^3$ exponentially growing HL60 cells were seeded into 96-well plates (3599; Corning Incorporated, USA). After 24 h, HL60 cells were fed with RPMI-1640 medium containing 0.1% fetal bovine serum and treated with gracillin (10 µl per well) at concentrations ranging from 0 to 100 µM and every kind cell was repeated in three holes. The plate was incubated at 37 °C for a further 24, 48, 72 h.

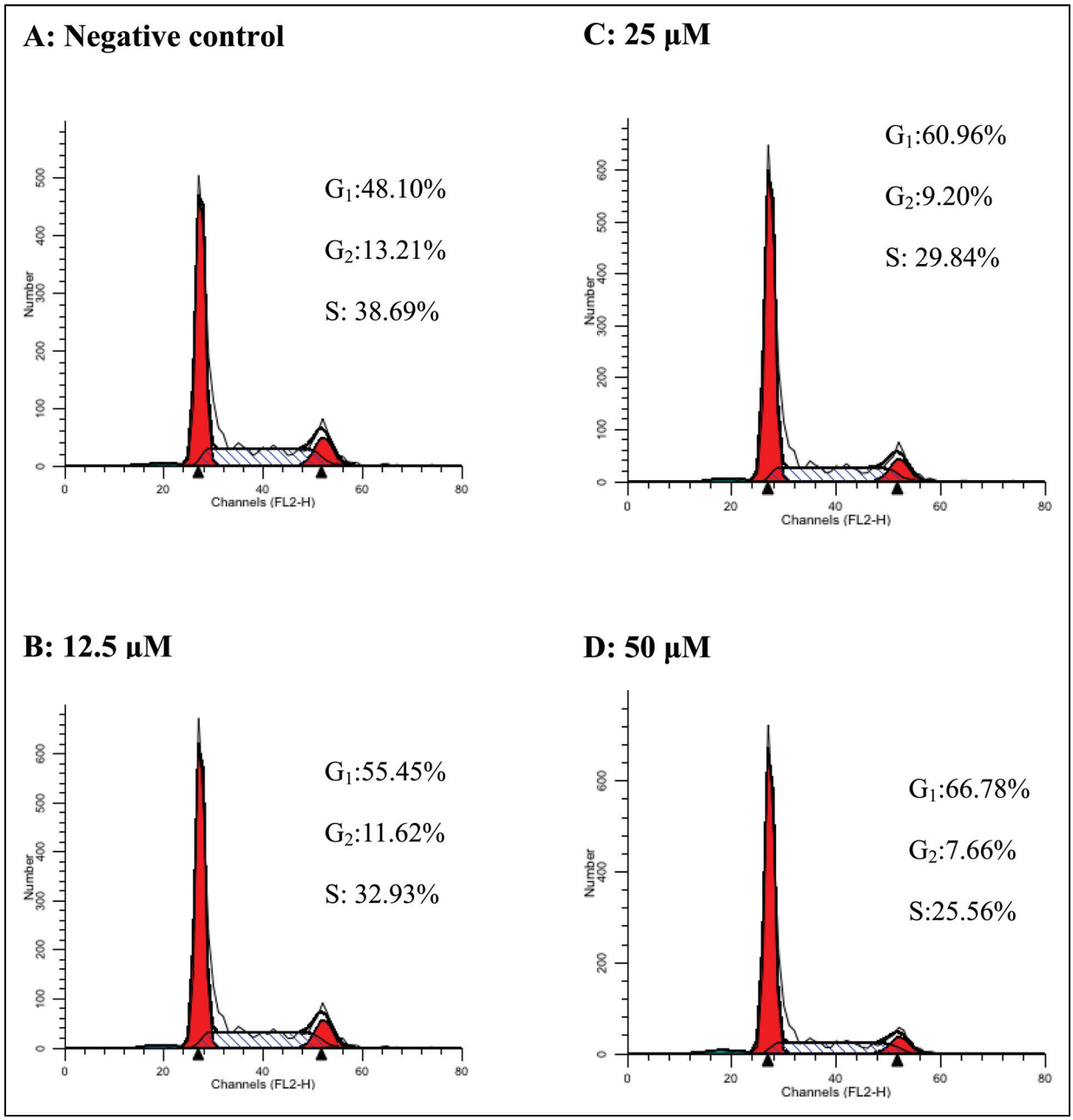

Fig. 4: Flow cytometric analysis of cell cycle distribution in HL60 cells which were treated with gracillin. A: Negative control HL-60 cells; B,C and D show cells treated with 12.5, 25 and 50 µM of gracillin for 48 h respectively.

10 µl of CCK-8 (KGA317; Nanjing KeyGen Biotech) was added to each well of the plate and the cells were incubated for an additional 4 h. The absorbance (A) of each well was measured at 450 nm using a microplate spectrophotometer (BIO-RAD xMark,USA). The inhibition ration of cell proliferation (IR) was calculated according to the following formula:
IR = (1-average A450 of the experimental group/average A450 of the control group) x 100%.

4.4. Apoptosis analysis

Apoptotic cells were quantified using the Annexin V-FITC apoptosis detection kit, according to the manufacturer's instructions.HL60 Cells (3×10^5) from exponential phase cultures were seeded in each well of a 6-well plate for 24 h. Then, the cells were treated with gracillin at concentrations of 12.5, 25 and 50 µM. Control untreated cells were also included. After incubation for 48 h, the cells were collected and washed twice with cold PBS. Next, The cell pellets were mixed with 100 µL of 1X binding buffer (Annexin V-FITC Apoptosis Detection Kit I (KGA105; Nanjing KeyGen Biotech, China). Subsequently, 5 µL of Annexin-V FITC and 10 µL of propidium iodide (PI) were added into the suspension and incubated for 15 min at 37 °C in the dark. Then 300 µL of 1X binding buffer was added before measurement. The number of viable, apoptotic and necrotic cells were quantified by flow cytometer (Becton Dickinson, USA) and analysis by the Cell Quest software. At least 10,000 cells were collected for each measurement in a triplicate experiment.

4.5. Cell cycle analysis

To analyse the cell cycle phase distribution, 3×10^5 of HL60 cells were seeded in each well of a 6-well plate for 24 h. Then, 12.5, 25 and 50 µM Gracillin were added to the cell samples respectively; PBS was added to the negative control group. After incubation for 48 h, cells were collected and fixed in 70% ethanol at 4 °C overnight. Ethanol was removed completely by centrifugation. Subsequently, cells were treated with 100 µl of RNase at 37 °C for 30 min and stained with 400 µl of PI (KGA511; Nanjing KeyGenBiotech) in the dark at 4 °C for 30 min. The cell cycle distribution was determined with a flow cytometer (Becton Dickinson, USA) using the Cell Quest Pro software (Becton Dickinson, USA). At least 10,000 cells were collected for each measurement in a triplicate experiment.

4.6. Assessment of oxidative damage

The severities of oxidative stress in the control and experimental groups of cells were evaluated by Malondialdehyde (MDA) content and super-

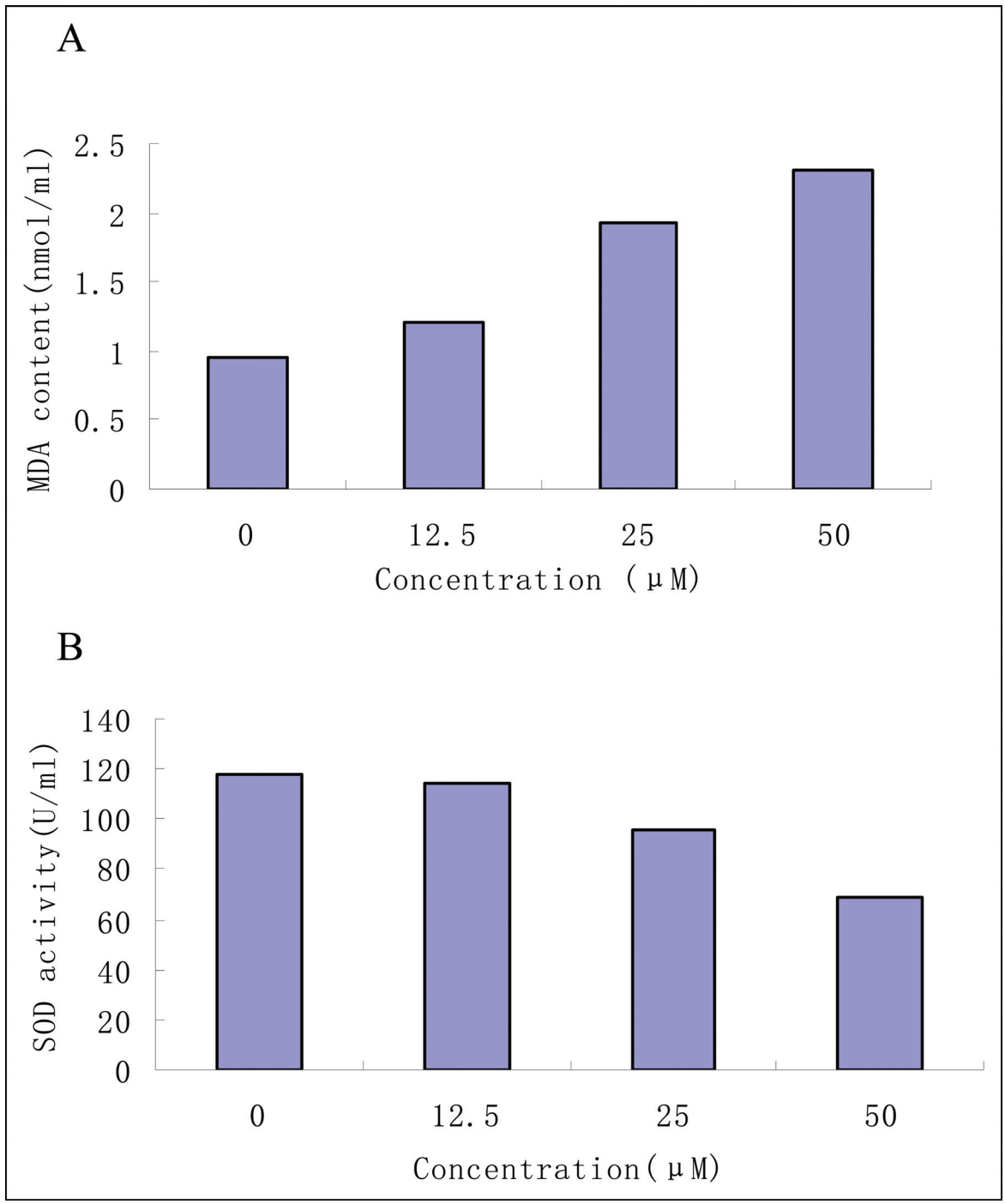

Fig. 5: Effect of gracillin on MDA content (A) and SOD activity (B) in HL60 cells. Gracillin treatment increased the content of MDA and declined SOD activity.

oxide dismutase (SOD) activity. The content of MDA and SOD activity were measured by using commercially available kits according to the manufacturer's instructions (KGT00150; Nanjing KeyGen Biotech, Nanjing, China). The activity of SOD was expressed as U/ml. MDA was measured at a wavelength of 532 nm by reacting with thiobarbituric acid to form a stable chromophoric production. The content of MDA was expressed as nmol/ml.

4.7. Real time-PCR analysis

The mRNA levels of Bcl-2 and Bax were measured in gracillin-deprived and control HL60 cells using real time-PCR. The primers used in the experiment are shown in the Table 1 and GAPDH was used as an internal control. HL60 Cells were seeded into 12-well plates at the density of 5×10^5 cells/well and cultured for 24 h. Then, the cells were treated with various concentrations of gracillin (12.5 μM, 25 μM, 50 μM) for 48 h. The total RNA from the cultured cells was isolated with TRIzol reagent according to the manufacturer's specifications (Invitrogen, USA). The extracted total RNA was subjected to cDNA synthesis using the Biorad iScriptTM Select cDNA Synthesis kit (Fermentas, Lithuania) containing random and oligo (dT) primer mix. The quantitative real time -PCR was performed on the ABI 7300 PCR System (Applied Biosystems, USA) using Power SYBR Green PCR Master Mix (ABI, USA). The thermal cycle profile was 95 °C for 5 min, followed by 40 cycles 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 40 s. The relative expression levels of target genes were obtained by the software SDS v1.3.2 attached with the PCR machine.mRNA expression of each target gene was normalized to GAPDH.

4.8. Statistical analysis

The data are expressed as means ± S.D. Analysis of variance was performed with SPSS software for windows 17.0 by using one way ANOVA and pairwise comparison with Student's t -test. The criterion for statistical significance was taken as $P < 0.05$.

Table 1: Sequences of the primers

genes	Primer Sequences		length
GAPDH	Forward primer	5- TGTTGCCATCAATGACCCCTT -3	202bp
	Reverse primer	5- CTCCACGACGTACTCAGCG -3	
Bcl-2	Forward primer	5- ATCCAGGATAACGGAGGC -3	81bp
	Reverse primer	5- CAGCCAGGAGAAATCAAAC -3	
Bax	Forward primer	5- GACCCGGTGCCTCAGGATGC -3	117bp
	Reverse primer	5- GTCTGTGTCCACGGCGGCAA -3	

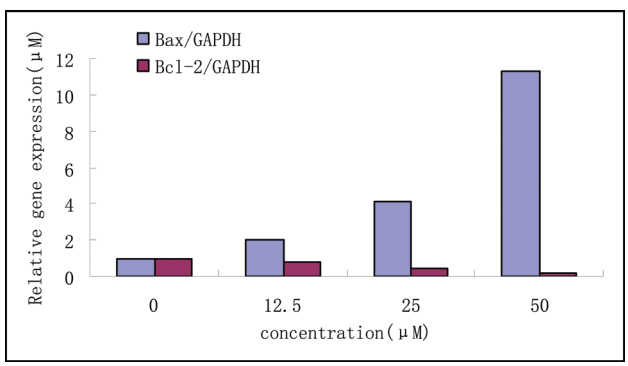

Fig. 6: Effects of gracillin on the expression of Bcl-2 and Bax in HL60 cells. Cells were treated with out or with 12.5, 25 μM, and50 μM of Gracillin for 48 h.The mRNA levels of Bcl-2 and Bax were measured by real time-PCR. Gracillin decreased the expression of Bcl-2 while increased the expression of Bax, the Bcl-2/Bax ratio was markedly down-regulated.

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References

Adams JM, Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281: 1322–1326.

Balsevich JJ, Ramirez-Erosa I, Hickie RA, Dunlop DM, Bishop GG, Deibert LK (2012) Antiproliferative activity of Saponaria vaccaria constituents and related compounds. Fitoterapia 83: 170–181.

Chen CR, Xia YH, Yao SY, Zhang Q, Wang Y, Ji ZN (2012) Virosecurinine induces apoptosis by affecting Bcl-2 and Bax expression in human colon cancer SW480 cells. Pharmazie 67: 351–354.

Coultas L, Strasser A (2003) The role of the Bcl-2 protein family in cancer. Sem Cancer Biol 13: 115–123.

Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. Cell 116: 205–219.

Deng JS, Chang YC, Wen CL, Liao JC, Hou WC, Amagaya S, Huang SS, Huang GJ (2012) Hepatoprotective effect of the ethanol extract of Vitis thunbergii on carbon tetrachloride-induced acute hepatotoxicity in rats through anti-oxidative activities. J Ethnopharmacol 142: 795–803.

Gerl R, Vaux DL (2005) Apoptosis in the development and treatment of cancer. Carcinogenesis 26: 263–270.

Gil P, Farinas F, Casado A, Lopez-Fernandez E (2002) Malondialdehyde: a possible marker of ageing. Gerontology 48: 209–214.

Hasnan J, Yusof MI, Damitri TD, Faridah AR, Adenan AS, Norbaini T H (2010) Relationship between apoptotic markers (Bax and Bcl-2) and biochemical markers in type 2 diabetes mellitus.Singapore Med J 51: 50–55.

Hou R, Zhou QL, Wang BX, Tashiro S, Onodera S, Ikejima T (2004) Diosgenin induces apoptosis in HeLa cells via activation of caspase pathway. Acta Pharmacol Sin 25: 1077–1082.

Hsieh MJ, Tsai TL, Hsieh YS, Wang CJ, Chiou HL (2013) Dioscin-induced autophagy mitigates cell apoptosis through modulation of PI3K/Akt and ERK and JNK signaling pathways in human lung cancer cell lines. Arch Toxicol 11: 1927–1937.

Ji ZN, Ye WC, Liu GG, Hsiao WL (2002) 23-Hydroxybetulinic acid-mediated apoptosis is accompanied by decreases in bcl-2 expression and telomerase activity in HL-60 Cells. Life Sci 72: 1–9.

Kaufmann SH, Gores GJ (2000) Apoptosis in cancer: cause and cure. BioEssays 22: 1007–1017.

Lecane PS, Karaman MW, Sirisawad M (2005) Motexafin gadolinium and zinc induce oxidative stress responses and apoptosis in B-cell lymphoma lines. Cancer Res 65: 11676–11688.

Newman DJ, Cragg GM (2012) Natural products as sources of new drugs from 1981 to 2010 J Nat Prod 70: 461–477.

Raju J, Mehta R (2009) Cancer chemopreventive and therapeutic effects of diosgenin, a food saponin. Nutrition Cancer 61: 27–35.

Senderowicz AM (2004) Targeting cell cycle and apoptosis for the treatment of human malignancies. Curr Opin Cell Biol 16: 670–678.

Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D (2005) Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. J Biol Chem 280: 19911–19924.

Srinivasan S, Koduru S, Kumar R, Venguswamy G, Kyprianou N, Damodaran C (2009) Diosgenin targets Akt-mediated prosurvival signaling in human breast cancer cells. Int J Cancer 125: 961–967.

Tang XP, Tang GD, Fang CY, Liang ZH, Zhang LY(2013) Effects of ginsenoside Rh2 on growth and migration of pancreatic cancer cells. World J Gastroenterol 19: 1582–1592.

Tor YS, Yazan LS, Foo JB, Armania N, Cheah YK, Abdullah R, Imam MU, Ismail N, Ismail M (2014) Induction of apoptosis through oxidative stress-related pathways in MCF-7, human breast cancer cells, by ethyl acetate extract of Dillenia suffruticosa. BMC Complement Altern Med 14: 55–66.

Wang CZ, Aung HH, Mehendale SR, Shoyama Y, Yuan CS (2010) High performance liquid chromatographic analysis and anticancer potential of Oplopanax horridus: comparison of stem and berry extracts. Fitoterapia 81: 132–139.

Wei Y, Xu Y, Han X, Qi Y, Xu L, Xu Y, Yin L, Sun H, Liu K, Peng J (2013) Anti-cancer effects of dioscin on three kinds of human lung cancer cell lines through inducing DNA damage and activating mitochondrial signal pathway. Food Chem Toxicol 59: 118–128.

Wong RSY (2011) Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 26: 30–87.

Wyllie AH (1997) Apoptosis: an overview. Br Med Bull 53: 451–465.

Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9:47–59.

Yu C, Huang X, Xu Y, Li H, Su J, Zhong J, Kang J, Liu Y, Sun L (2013) Lysosome dysfunction enhances oxidative stress-induced apoptosis through ubiquitinated protein accumulation in HeLa cells. Anat Rec (Hoboken) 296: 31–39.

Zhang JY, Tao LY, Liang YJ, Yan YY, Dai CL, Xia XK, She ZG, Lin YC, Fu LW (2009) Secalonic acid D induced leukemia cell apoptosis and cell cycle arrest of G1 with involvement of GSK-3β/β-catenin/c-Myc pathway. Cell Cycle 15: 2444–2450.