

Effects of scabraside D on cell viability inhibition and apoptosis promotion of human hepatocellular carcinoma

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Abstract

Scabraside D, derived from the sea cucumber *Holothuria scabra* is a sulfated triterpene glycoside which possess in various biological activities. We assessed the activity of scabraside D and their effects on cell viability and apoptosis on human hepatocellular carcinoma (HepG2) cell lines by MTT assay and staining with Hoechst 33342. The 25 to 100 µg/mL dose of scabraside D significantly decreased the viability of HepG2 cells, in a dose-dependent manner. The treatment with scabraside D at dose 50 and 100 µg/mL significantly also induces morphological changes of apoptotic cell, including cell shrinkage, nuclear chromatin condensation and nuclear fragmentation. Quantitative real-time PCR shows that scabraside D up-regulated *Bax* and *Caspase-3* while down-regulated *Bcl-2* expressions in the HepG2 cells in dose-dependent manner. In conclusion, scabraside D can inhibit cell viability and induce apoptosis in HepG2 cells. This study show that scabraside D may be used as a new therapeutic agent for human hepatocellular carcinoma

Keywords: scabraside D, hepatocellular carcinoma, cell viability, apoptosis

ประสิทธิภาพของสคาบราไซด์ ดี ต่อการยับยั้งการมีชีวิตและสนับสนุนให้เกิดการตายแบบอะพอพโทซิสของเซลล์มะเร็งตับมนุษย์

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บทคัดย่อ

สคาบราไซด์ ดี (scabraside D) สกัดได้จากปลิงทะเลขาว (*Holothuria scabra*) คือสาร ซัลเฟตเตต ไตรเทอปีน ไกลโคไซด์ สารสกัดนี้ได้แสดงฤทธิ์ทางชีวภาพหลากหลาย การศึกษาครั้งนี้จะประเมินการทำงานของ สคาบราไซด์ ดี และผลต่อการมีชีวิตอยู่ และการตายแบบอะพอพโทซิสต่อเซลล์มะเร็งตับมนุษย์ (HepG2) โดยวิธี MTT assay และย้อมด้วย Hoechst 33342 พบว่า สคาบราไซด์ ดี ขนาด 25-100 ไมโครกรัม/มิลลิลิตร สามารถลดการมีชีวิตของเซลล์มะเร็งตับมนุษย์ได้อย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังพบว่า สคาบราไซด์ ดี จะเหนี่ยวนำให้เกิดการตายแบบ อะพอพโทซิส เช่น เซลล์มีลักษณะหดตัว มีการหดตัวและแตกหักของสายโครมาติน ขณะที่ผลของปฏิกิริยาถูกโซ่พอลิเมอเรสแบบเรียลไทม์ (Quantitative real-time PCR) แสดงให้เห็นสคาบราไซด์ ดี ที่ความเข้มข้น 50 และ 100 ไมโครกรัม/มิลลิลิตร ส่งผลให้มีการ เพิ่มระดับของยีน *Bax* และ *caspase-3* แต่ลดการแสดงออกของยีน *Bcl-2* ในเซลล์มะเร็งตับของมนุษย์ สรุปผลการทดลองพบว่า สคาบราไซด์ ดี สามารถยับยั้งการมีชีวิตของเซลล์มะเร็งตับมนุษย์และเหนี่ยวนำให้เกิดการตายของเซลล์แบบอะพอพโทซิส (apoptosis) อย่างมีนัยสำคัญทางสถิติ การศึกษาครั้งนี้แสดงให้เห็นว่า สคาบราไซด์ ดี อาจสามารถใช้เป็นสารใหม่ที่ใช้ในการรักษามะเร็งตับในมนุษย์ได้

คำสำคัญ : สคาบราไซด์ ดี, ปลิงทะเล, มะเร็งตับมนุษย์, การมีชีวิตอยู่, การตายแบบอะพอพโทซิส

Introduction

Hepatocellular carcinoma (HCC), a primary cancer of the liver is caused by mutation in hepatocytes (Srivatanakul et al., 2004; Aravalli, et al., 2008). It is the fifth most common cancer and the third leading cause of cancer death worldwide. The chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is major courses of HCC. HCC is a cancer that occurs often in several developing countries, and has been found that it is associated with many risk factors. In Thailand the high incidence of HBV associated with HCC has been reported. The infection is transmitted from mothers to their infants, up to 90% of infected people. Thus chronic infection with HBV is the major cause of HCC in this area (Srivatanakul et al., 2004). Chemotherapy of HCC is mainly ineffectual and clinical potency of regular treatment with 5-fluorouracil (5-FU) is low (Henjina et al., 1995). Recently, therapeutics of HCC with chemotherapy and radiotherapy cause severe side effects. Therefore, the discovery and development of an effective treatment are desired. Natural products have contributed essentially to the treatment of cancer. (Rajasekaran et al., 2008). One of the significant anticancer therapy is to induce apoptosis in cancer cells.

Apoptosis is programmed cell death, that crucial role in cellular homeostasis. Eccentric cells are removed by apoptosis, whereas cancer cells progress mechanisms to protect this process just as they manipulate to escape cellular growth restriction (Okada and Mak, 2004; Ghobrial et al., 2005). The mechanisms of apoptosis composed of two main pathways including the extrinsic or death receptor pathway and intrinsic or mitochondrial pathway (Wong, 2011). Apoptosis is mediated by stimulation of a cascade of serine protease, known as caspase. There are initiator caspase and executioner

caspase. Initiator caspase consists of caspase 8 and 9 that stimulus the cascade, while executioner or effector caspase consists of caspase 3, 6, 7 and others that are amenable for cleavage of substrates (Okada and Mak, 2004; Ghobrial et al., 2005). The extrinsic pathway, involves binding of tumor necrosis factor (TNF), Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL) to their sequent particular receptors, which divide common internal region known as death domain. Ligand binding causes receptor trimerization and recruiting of Fas-associated death domain protein (FADD) and caspase-8 to produce the death-inducing signaling complex (DISC), after that caspase-8 is stimulated by cleavage (Elnemr et al., 2001). The intrinsic pathway is begun within the cell. This pathway result in increased mitochondrial permeability and release of cytochrome-c (pro-apoptotic molecules) into cytoplasm. Beside, this pathway is closely controlled by a group of proteins belonging to the Bcl-2 family. There are two major groups of Bcl-2 proteins, including pro-apoptotic protein (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic protein (Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1). The pro-apoptotic protein can promote the mitochondria release of cytochrome-c into the cytosol. Meanwhile, the anti-apoptotic protein control apoptosis by inhibiting the mitochondrial release cytochrome c (Green and Reed, 1998; Hengartner, 2000). When cytochrome c release into the cytoplasm and activates caspase-3 leading to cell apoptosis. This process leads to DNA fragmentation and cell destruction (Kaufmann and Gores, 2000; Wong, 2011; Elmore, 2007). Apoptosis is characterized by membrane blebbing, the appearance of extremely condensed chromatin and stimulation of an endonucleolytic process, which conduce to the subsequent cleavage of chromosomal DNA. As a result, cell shrink

and condense into apoptotic bodies (Wu et al., 2001; Berthiaume and Wands, 2004; Okada and Mak, 2004; Wong, 2011).

Triterpene glycosides which are found in the plant species and marine animals. Source of triterpene glycosides have been found in many legumes for example soyabeans, beans, peas, etc. and also found in alliums, liquorice, quinoa, spinach, sugar beet, tea, ginseng, sunflower, and horse chestnut. Moreover, triterpene glycosides can be found in marine animals which have only been found in the marine phylum Echinodermata (Hostettmann and Marston, 1995). Sea cucumbers, *Holothuria scabra* (*H. scabra*) as one of marine animal, which is a potent source for new sort of biologically active substances with medical application (Kerr and Chen, 1995). Triterpene glycosides extracted from sea cucumber becomes increasing scientific interest in pharmacology and ecology (Caulier et al., 2011). Its various biological activities has been reported including, antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory effects (Kitagawa et al., 1989; Stonix et al., 1999; Chludil et al., 2002; Zhang^a, 2011; Zhang^b, 2011). One of the bioactive substances is scabraside D which is a new sulfated triterpenoid glycoside isolated from *H. scabra*. This agent could inhibit the proliferation some types of cancer cells and induce apoptosis in human cholangiocarcinoma cells (Han et al., 2012; Assawasuparerk et al., 2016). However, its effects on human hepatocellular carcinoma (HepG2) cells has not been previously determined. But the mechanisms of action of scabraside D on HepG2 cells are still unknown, this study aimed to investigate the effects of scabraside D on HepG2 cell viability and apoptosis promotion *in vitro*.

Material and Methods

Chemicals and reagents

Scabraside D ($C_{54}H_{87}O_{27}SNa_2$) was obtained from Dr. Saksit Nobsathian, Nakornsawan Campus, Mahidol University. Minimum essential medium (MEM) was purchased from Invitrogen-Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) were purchased from Hyclone USA. Dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Hoechst 33342, 5-fluorouracil (5-FU) were purchased from Sigma Inc., USA. Other common reagents were obtained from the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University Thailand.

Cell lines and cell culture

Human hepatocellular carcinoma cell line (HepG2) and baby hamster kidney fibroblast cell line (BHK-21) fibroblast cell line were obtained from the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University. The cells were cultured in MEM containing 10% FBS, penicillin (100 IU/mL) and streptomycin (100 / mL). HepG2 cell line was routinely maintained at 37°C in an incubator with a humidified atmosphere of 95% air and 5% CO₂.

Cell viability assay

The cell viability was defined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. This assay was depends on the capability of succinate dehydrogenase enzyme in living mitochondrial to separate the yellow tetrazolium salt MTT into the blue

or purple insoluble formazan product (Mosmann, 1983) that can be read spectrophotometrically. HepG2 cells and BHK-21 cells were incubated in 96-well plates at the density of 1×10^4 cells/well overnight. The cells were then treated with scabraside D the different concentration of either scabraside D or 5-FU (6.25, 12.5, 25, 50, 100 $\mu\text{g}/\text{mL}$) control (0 $\mu\text{g}/\text{mL}$) and were incubated for 24 h. The 100 μL of MTT solution (0.5 mg/mL) was subsequently added into each well and the solution was incubated for 4 h at 37°C of incubation in dark (Christy et al., 2007). After 24 h, the media was discarded and cells were washed with serum-free media. Then, 100 μL of DMSO was added into each well to solubilize the blue formazan products and incubated for 5 min in dark at room temperature. Cytotoxicity was determined spectrophotometrically at OD 490 nm using microplate reader (Bio-Rad, Hercules, CA). The cytotoxicity was presented as 50% inhibition concentration (IC_{50}) or concentration that causes 50% loss of cell viability according to the equation: % Inhibition = [(A490 control cells - A490 treated cells) / A490 control cell $\times$ 100]. The mean IC_{50} was determined from at least three independent tests. The averaged values were calculated from at least three independent experiments, and were presented as percentages of cell viability.

Apoptosis of HepG2 cells following scabraside D treatment

Morphological analysis

The nuclear characteristics of HepG2 cell after the treatment with scabraside D was observed, nuclear staining with Hoechst 33342. The HepG2 cells (5×10^5 cells) were treated with various concentrations of scabraside D and 0 $\mu\text{g}/\text{mL}$ (control) for 24 h. Then the cells were washed two times with cold phosphate-buffered saline

(PBS), and then fixed with cold methanol and acetic acid (3/1, v/v) at 4°C overnight. Next, the cells were incubated with Hoechst 33342 for 30 min in the dark. After that, they were mounted and evaluated under a Confocal Laser Scanning microscope (Olympus FV 1000, Japan).

Quantitative real-time polymerase chain reaction (Quantitative real-time PCR)

Quantitative real-time PCR using define the exposition levels of *Bcl-2*, *Bax*, *caspase-3* and *GAPDH* were performed in KAPA SYBR® FAST qPCR kit Master Mix (2X) (KAPA BIOSYSTEMS, USA), and was processed in an ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA). The specific primers were designed according to the full cDNA sequence of *Bax* (accession AY217036), *Bcl-2* (accession BC027258), *caspase-3* (accession NM_004346), and *GAPDH* (accession NM_002046) using Primer Blast Program (Ye et al., 2012). The following specific primers (Pacific Science Co., Ltd.) were used, i.e. *Bax*, Forward: 5'-ATGCCCCGTTTCATCTCAGTCC-3', Reverse: 5'-GAAGTGTGTCCCGAAGGAGG-3'; *Bcl2*, Forward: 5'TGGGAGAACGGGGTACGATA-3', Reverse: 5'-CATGACCCCAACGAACTCAA-3'; *caspase3*, Forward: 5'GTTGGCGTCGCCTTGAAATC-3', Reverse: 5'TGAGGTTTGCTGCATCGACA3'; *GAPDH*, Forward: 5'CTCCTGTTCGACAGTCAGCC-3', Reverse: 5'-TTCCCGTTCTCAGCCTTGAC-3'. The conditions were as follows: the amplification primary incubation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 10 min, annealing at 60°C for 1 min, and extension at 60°C for 1 min. The sequences of these PCR products were harvested by direct sequencing. Each amplicon were cloned into the PGEM®-t Vector (Promega, Madison, WI, USA) in order to establish standard curves for target

cDNA. The mRNA level was informed as the ratios of the copy numbers of target cDNA to GAPDH cDNA. The ratio of fold alter in gene exposition level between the treatment and control cells is defined to identify the up down control of gene exposition.

Statistical analysis

Data of cell viability and quantitative real-time PCR were presented as mean $\pm$ SD from three or more independent experiments. Statistically significance was calculated by student's t-test and analysis of variance (ANOVA) followed by Bonferroni post-test in GraphPad Prism program version 5 (GraphPad software, San Diego, CA). All data were considered statistically significant at p-values < 0.05 .

Results

Inhibition of HepG2 cell proliferation

The cell viability of HepG2 cells treated with scabraside D decreased in a dose dependent-manner at 24 h (Figure 1A). The mean concentration of 50% inhibitory concentrations (IC_{50}) on HepG2 cell was 55.76 ± 0.255 $\mu\text{g/mL}$. In contrast, BHK-21 treated with scabraside D at the concentration from 25 to 100 was higher than 50% cell viability (Figure 1B). The treatment with scabraside D statistically significant inhibit HepG2 cell viability. In addition, the viability of HepG2 cell treated with scabraside D was comparable to 5-FU. The result presented that the treatment with scabraside D and 5-FU has similar effects on the reduction of HepG2 cell viability (Figure 2).

Scabraside D induce apoptosis in HepG2 cells

To evaluate the morphological changes of the cell's nucleus treated with scabraside D. Hoechst fluorescent

dye was used to stain cell nucleus and examined under Confocal Laser Scanning microscope. The results showed that treated cells appeared as shrunken cells with condensed nuclear chromatin, and as fragmented nuclei (Figure 3B) whereas all untreated cells exhibit round nucleus and long process (Figure 3A).

Effect of scabraside D on the expression of apoptosis-related genes in HepG2 cells

The results indicated that treatment with scabraside D enhanced *Bax* and *caspase-3* (Figure 4A and Figure 4D) but reduced *Bcl-2* levels in HepG2 cells in a dose-dependent manner when compared with control (Figure 4B). These resulted in an increasing ratio of *Bax/Bcl-2* mRNA in the scabraside D-treated group in a dose-dependent manner (Figure 4C). In all the values obtained, significant differences to the no treatment group ($p < 0.05$) were presented when the dose of scabraside D treated with HepG2 cells were significant difference at 50 and 100 $\mu\text{g/mL}$. In all values obtained, scabraside D at dose 50 and 100 $\mu\text{g/mL}$ significantly enhanced *Bax* and *caspase-3* but reduced *Bcl-2* levels compared to other doses

Discussion

Nowadays, the utilization of natural products for cancer protection and treatment has acquired a great deal of concentration owing to their different health advantage and prominent lack of toxicity and side effects. Sea cucumbers are significant elements of traditional Chinese medicine and healthy supplement food. Active compounds extracted from sea cucumbers have interesting biological activities with possibility for clinical use. One group of active compounds is triterpene glycosides which has been shown many biological activities, for example antifungal,

cytotoxic, hemolytic, cytostatic and immunomodulatory effects (Habermehl and Volkwein, 1971). In addition, triterpene glycosides isolated from mushroom *Fomitopsis nigra* and sea cucumber *H. scabra* can inhibit cancer cells have similar effects. Mechanism of action of triterpene glycosides has been found to induce apoptosis of human oral squamous cell carcinoma and human cholangiocarcinoma cells via decrease of *Bcl-2* and increase of *Bax* which result in the stimulation of *caspase-3* (Bhattarai et al., 2012 and Assawasuparerk et al., 2016). Scabraside D, which was derived from *H. scabra*, has a structure as a new sulfated triterpenoid glycoside. It shows cytotoxicity against six types cancer cell lines including, mouse leukemic cell (P-388), human lung cancer cell (A-549), gastric cancer cell (MKN-28), human colorectal cancer cell (HCT-116) and human breast cancer cell (MCF-7) and human cholangiocarcinoma cell (HuCCA) (Habermehl and Volkwein, 1971; Assawasuparerk et al., 2016). The mechanism of action of scabraside D on HepG2 cells have not previously reported and this is the first study that we examined its mechanisms of action on HepG2 cells. The results showing the decrease in cell viability following the scabraside D treatment specifies the toxicity of the scabraside D on HepG2 cells. However, no toxicity of scabraside D on BHK-21 cells was demonstrated. Thus, the scabraside D was able to inhibit cancer cell viability while there was no toxicity affected to normal fibroblast. Moreover, the data also showed that both scabraside D and 5-FU displays the similar action on the decrease in HepG2 cell viability at the same concentration. 5-FU is extensively used as anticancer. Earlier, a report found that 5-FU treated cancer cells experienced necrosis, which had affect to normal cells (Chen et al., 2014), whereas this study showed that apoptosis was induced following

treating with the scabraside D. The capability to lead apoptosis in cancer cells, without affecting normal cells, as well as reducing side effects, are important goals for the development of novel anticancer drugs. The effect of scabraside D on viability inhibition of HepG2 cells was similar to those in the previous research by Assawasuparerk et al., 2016 (Assawasuparerk et al., 2016). HepG2 cells were likely modulated through apoptosis by which apoptotic morphology include cell shrinkage, chromatin condensation and nuclear fragmentation. Besides, this research examined the expression levels of apoptosis related-genes in the scabraside D treated cells, presented increase of *caspase-3*, *Bax* and decrease of *Bcl-2*, result in enhance in *Bax/Bcl-2* ratio. The *Bcl-2* family plays an important role in apoptotic pathway via the mitochondrial pathway (Green and Reed, 1998; Oltersdorf et al., 2005). The mitochondrial pathway is precious induction of disruption in mitochondria through the modification of *Bax/Bcl-2* ratio. Increasing *Bax* activate the intrinsic apoptotic pathway by inducing permeabilization of the outer mitochondrial membrane, resulting in a release of cytochrome C into the cytoplasm and afterward caspase up regulation, whereas *Bcl-2* down regulation (Li et al., 2008; Tilli et al., 2002; Camins et al., 2008). The release of cytochrome c from mitochondria directly activates caspase-9-induced *caspase-3* activation. The *caspase-3* plays an important role in modification the nuclear DNA in apoptotic cells. (Tilli et al., 2002; Woo et al., 1998; Bras et al., 2005). Hence, it was clear that an increased number of apoptotic cells after scabraside D treatment was a cause of the decrease in HepG2 cell viability. In this research, increase of the *Bax*, *caspase-3* level and decrease of *Bcl-2* level, leading to increase *Bax/Bcl-2* ratio in the scabraside D treatment group may promote HepG2 cells apoptosis.

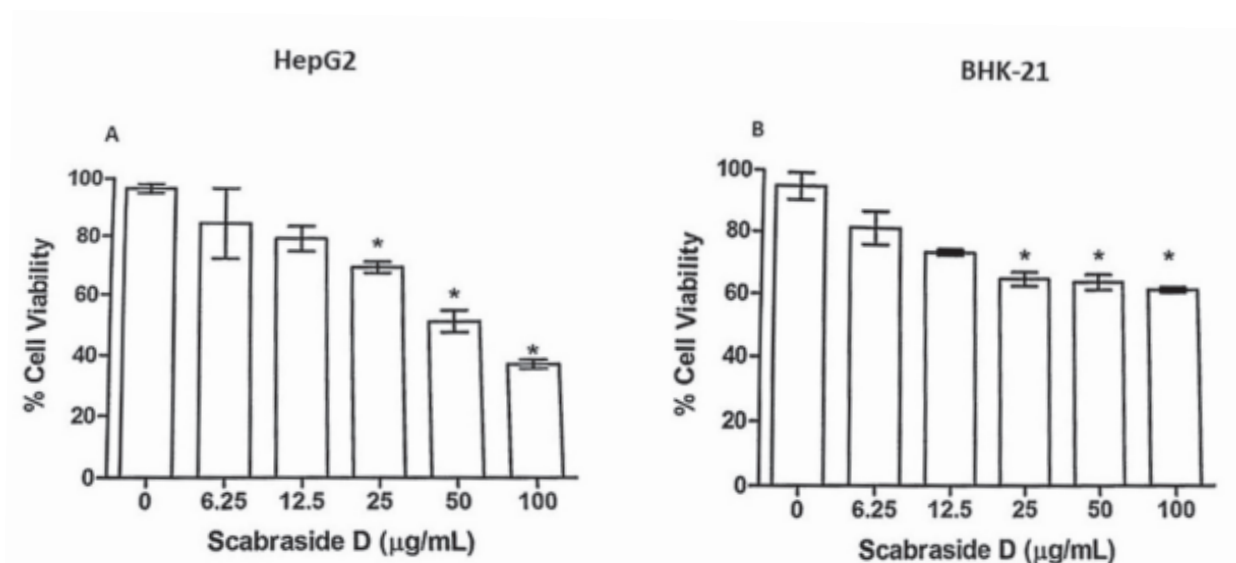


Figure 1. The effect of scabraside D on HepG2 (A) and BHK-21 (B) cell viability by MTT assay at 24 h. All values are mean $\pm$ SD (n=3). *, Statistical significant difference with $p < 0.05$ compared to control (0 µg/mL).

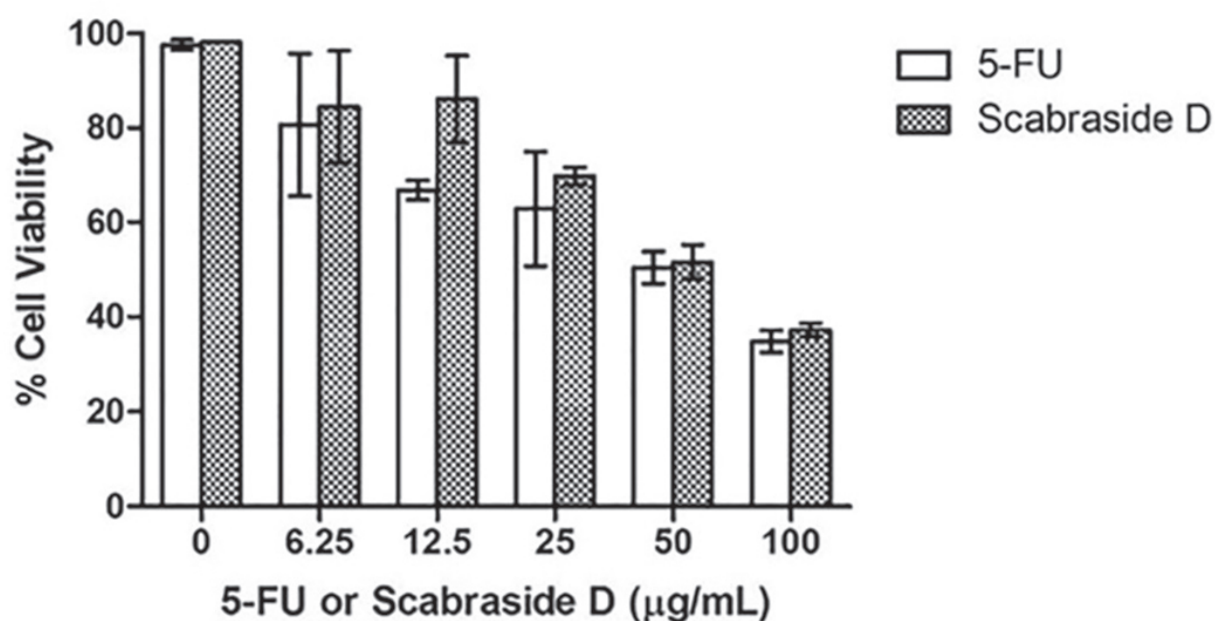


Figure 2. The effect of scabraside D and 5-FU on HepG2 cell viability evaluated by MTT assay. Scabraside D decreased cell viability relatively to 5-FU at the same dosage. All values are mean $\pm$ SD (n = 3).

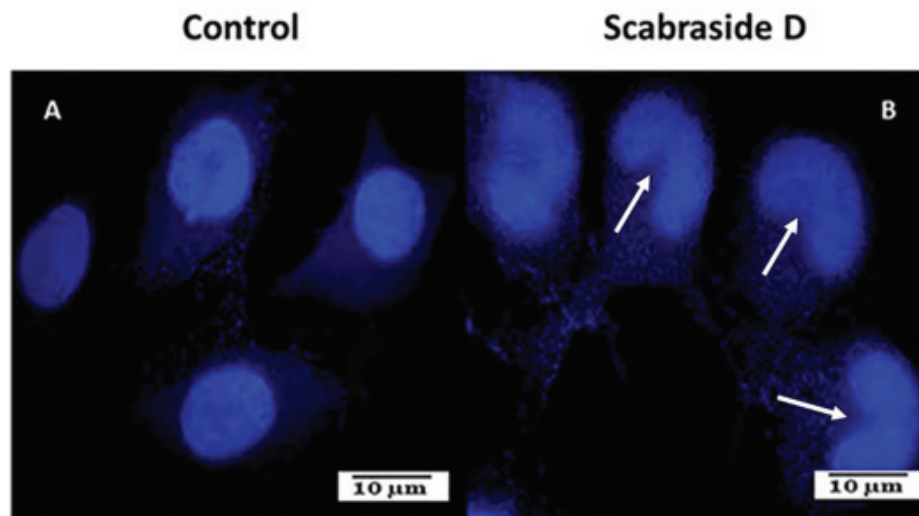


Figure 3. Microscopic morphology of HepG2 cells by Hoechst 33342 staining. Control group (A) and scabraside D treatment group (B). The scabraside D treatment HepG2 cells showed cellular shrinkage, chromatin condensation and nuclei fragmentation (arrow).

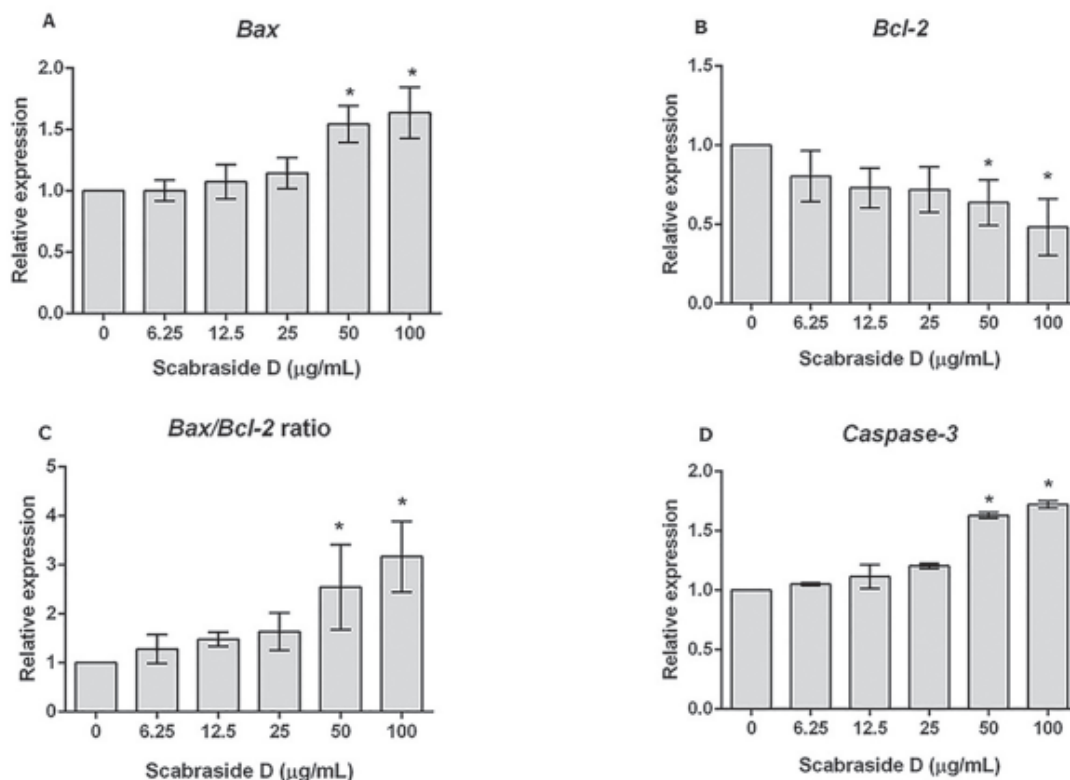


Figure 4. Quantitative real-time PCR reaction for gene expression in HepG2 cells. Scabraside D induced up-regulation of *Bax* (A) and *caspase-3* (D) and down-regulation of *Bcl-2* gene levels (B). The histograms of scabraside D treated HepG2 cells was used as a substitute; *Bax/Bcl-2* ratio (C). Data were expressed as mean $\pm$ SD relative to the control (0 µg/mL) values, and normalized base on *GAPDH* (n = 3).*, Statistical significant, p value < 0.05 vs treated group.

Conclusion

In conclusion, our study demonstrated that the scabraside D could inhibit the viability of HepG2 cell by the induction of apoptosis in dose-dependent manner. This apoptotic response was related with the increase of *Bax* to *Bcl-2* ratio and induce activation of *caspase-3*. Taken together, scabraside D might be used as a promising agent as a result of its effective viability inhibitor of human hepatocellular carcinoma cell and no toxicity as evaluating on normal fibroblast cells. Therefore, scabraside D had the potential to be developed as substance for the hepatocellular carcinoma therapy.

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