Taccaoside induces apoptosis in hepatocellular carcinoma cell lines

Yuewen Sun, Hanchen Qiu, Mingchun Ou, Runli Chen and Gang Liang

Abstract

Objective: Taccaoside, a steroidal saponin, has been shown to be cytotoxic, although the mechanism of cytotoxicity remains unclear. This study examined the effect of taccaoside on the human hepatocellular carcinoma (HCC) cell lines SMMC-7721 and Bel-7404.

Methods: The antiproliferative effect of taccaoside were measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Cells were stained with Hoechst 33258 to observe morphology. Cell cycle and apoptosis were analysed by flow cytometry. Caspase activation was detected using specific assays, and PARP, Bax and Bcl-2 expression were analysed using western blotting.

Results: Taccaoside showed antiproliferative effect on HCC cell lines growth in a concentration- and time-dependent manner. Taccaoside arrested cell cycle in the G2/M phase and induced caspase-dependent apoptosis. Western blotting indicated that taccaoside upregulated Bax expression and downregulated Bcl-2 expression. PARP cleavage was observed following taccaoside treatment.

Conclusions: This study showed that taccaoside may inhibit HCC cell proliferation by inducing apoptosis.

Introduction

Taccaoside (Figure 1) is a steroidal saponin derived from members of the *Taccaceae* family,[1] which is composed of two genera, *Tacca* and *Schizocapsa*. While members of the *Taccaceae* family have long been used to treat tumours and swelling in China,[2,3] the mechanism behind their anticancer properties is not well understood. Extensive

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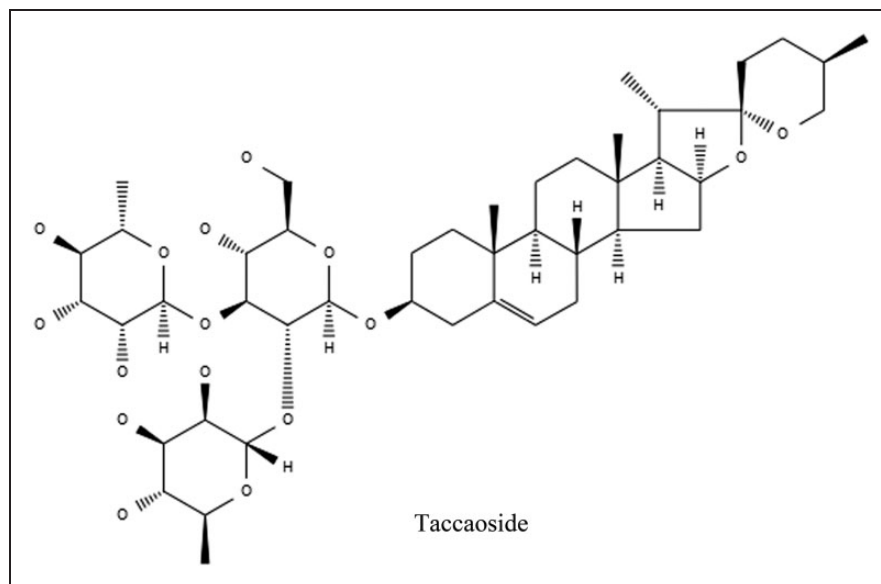Email: foreliane@163.com

Figure 1. The structure of taccaoside.

investigations into the *Tacca* family have identified potential active compounds,[4] and previous studies have demonstrated the cytotoxicity of *Taccaoside* and its structural analogues in hepatocellular carcinoma (HCC) cell lines,[5,6] although the specific mechanisms of action of *Taccaoside* remain elusive.

HCC is a common solid organ cancer with poor prognosis and high mortality.[7] Therapies for most HCC patients are inefficient because of chemotherapy resistance and the toxicity of chemotherapy agents.[8] In previous studies, several natural products have shown noteworthy anticancer properties, indicating that natural products may represent an important reservoir for anti-HCC drug screening.[9]

The aim of this study was to elucidate the antiproliferative mechanism of taccaoside in HCC cells.

Materials and methods

Materials and cell lines

Taccaoside was purified from *Schizocapsa plantaginea* (Hance) tubers according to the literature.[10] Human HCC cell lines SMMC-7721 and Bel-7404 were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Antibodies for β-actin, Bax, Bcl-2 and PARP were purchased from Abcam (Cambridge, UK).

Cell culture

Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Solarbio Science and Technology, Beijing, China) containing 15% foetal bovine serum (FBS) (Gibco®, Life Technologies, Carlsbad, CA, USA) at 37°C.

Cell viability analyses

SMMC-7721 and Bel-7404 cells were seeded into 96-well plates $(1 \times 10^4$ cells/well). Taccaoside was added to the cells at various concentrations, and each dosage group was repeated four times. Cell viability following drug exposure was determined using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Solarbio Science and Technology).

Hoechst 33258 staining

SMMC-7721 and Bel-7404 cells in the logarithmic growth phase were seeded into 6-well plates (2×10^5 cells/well). After 12 h, various concentrations of taccaoside were added to the wells. After a further 24 h of incubation, media was removed and cells were washed three times with phosphate buffered saline (PBS) and stained with 2 µg/ml Hoechst 33258 (Sigma-Aldrich, Poznan, Poland) at room temperature for 30 min.

Flow cytometry detection of apoptosis and cell cycle

Cells were trypsinised 24 h after taccaoside treatment and washed twice with PBS. Flow cytometry for cell cycle determination was performed using DNA PREP and DNA PREP stain according to the manufacturer's instructions (Beckman, Fullerton, CA, USA) or for apoptosis using a PE/7-AAD double-staining kit according to the manufacturer's instructions (Beckman). All experiments were repeated three times.

Caspase assays

SMMC-7721 and Bel-7404 cells were treated with taccaoside for 24 h and caspase activity was measure using a caspase activity assay according to the manufacturer's instructions (Beyotime, China).

Western blot

SMMC-7721 and Bel-7404 cells in the logarithmic growth phase were seeded into 6-well plates (5×10^5 cells/well). After 12 h, SMMC-7721 cells were treated with Taccaoside for 24 h. Scraped and collected the cells. Total cellular proteins were extracted with RIPA buffer (Beyotime, China) and quantified using a bicinchoninic acid (BCA) assay (Beyotime, China). Equal amounts of protein per sample were loaded onto SDS-PAGE gels and electrophoresed at

80 V for 30 min. Samples were then electrophoresed in a separation gel at 120 V for at least 90 min. Separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and blocked with 5% milk at room temperature for 1 h. Membranes were incubated overnight with primary antibodies with shaking at 4°C and then with secondary antibodies at room temperature for 2 h. All immunoblots were visualized by electrochemiluminescence using ECL Western Blotting Substrate (Solarbio, China). β-actin, Bax, Bcl-2 and PARP were diluted with 2% BSA (Solarbio, China) (1:1000). Membranes were washed with PBST (0.1% Tween) (PH7.5) for 5 times after primary and secondary antibody.

Statistical analysis

Experimental data were analysed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) with significance set at $P < 0.05$. Between-group differences were analysed using one-way analysis of variance (ANOVA) with the Bonferroni t-test. Data were presented as the mean $\pm$ SD.

Results

Taccaoside significantly inhibited the proliferation of SCCM-7721 and Bel-7404 cells in a time-concentration dependent manner (Figure 2) ($P < 0.01$). The IC50 of taccaoside in SMMC-7721 cells at 24 and 48 h was 2.55 and 1.72 µM, respectively, and in BEL-7404 cells at 24 and 48 h was 8.10 and 5.94 µM, respectively. Furthermore, cell density was markedly reduced in cells treated with taccaoside.

To investigate whether the antiproliferation effect of taccaoside were associated with the induction of apoptosis, cellular morphological changes were observed using Hoechst 33258 staining. Following treatment with 4 µM of taccaoside for 24 h, cytoplasmic blebbing, chromatin condensation and
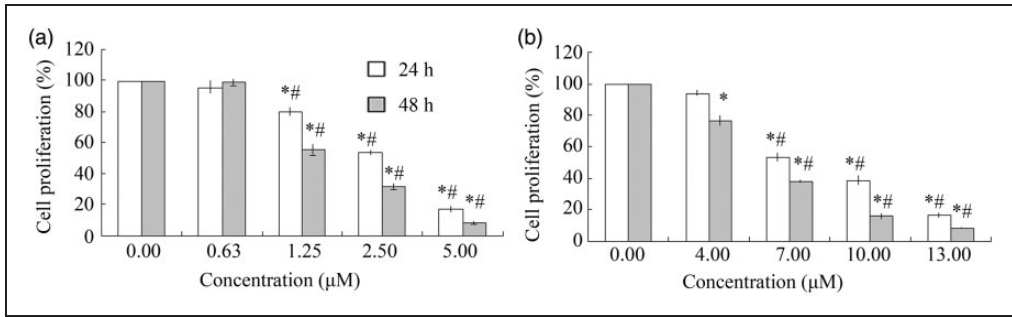
Figure 2. Effect of taccaoside on hepatocellular carcinoma (HCC) cell proliferation. Taccaoside significantly inhibited HCC cell proliferation. (a) SMMC-7721 cells were treated with taccaoside (0.63, 1.25, 2.50 and 5.00 μM) for 24 and 48 h. (b) Bel-7404 cells were treated with taccaoside (4.0, 7.0, 10.0 and 13.0 μM) for 24 and 48 h. Data are presented as the mean ± standard deviation (SD) ($n = 4$). *$P < 0.01$ compared with control group; #$P < 0.01$ compared with lowest dosage group.

nuclear fragmentation could be observed under a fluorescent microscope (Figure 3a).

PE-7AAD double staining was using to detect taccaoside-induced apoptosis. After exposure to taccaoside for 24 h, the apoptosis rates of Bel-7404 and SMMC-7721 cells were increased by 56.8% and 70.1%, respectively (Figure 3b). The increase in apoptosis was significantly dosage-dependent in both cell lines ($P < 0.01$) (Figure 3c). These results demonstrated that taccaoside inhibited cell proliferation by inducing apoptosis.

Cell cycle arrest also played an important role in the antiproliferation effect of taccaoside. The percentage of SMMC-7721 and Bel-7404 cells in the G2/M phase was significantly increased after treatment with taccaoside for 24 h ($P < 0.01$). For SMMC-7721 cells, the percentage of G2/M cells in the experimental group was 26.17%, with the percentage in the control group at 14.60%. For Bel-7404 cells, 23.60% of cells in the G2/M phase in the taccaoside group but only 13.77% of cells were in G2/M phase in the control group (Figure 3). Taccaoside treatment also increased the percentage of S phase cells ($P < 0.01$). The percentage of G0/G1 phase cells was significantly decreased following taccaoside treatment (Figure 4). ($P < 0.01$).

To determine the possible mechanism by which taccaoside induced apoptosis in HCC cells, western blot assays were used to detect the expression of apoptosis-related proteins. We found that taccaoside activated caspase-3 and -8 in both SMMC-7721 and Bel-7404 cells in a concentration-dependent manner (Figure 5) ($P < 0.01$). These results indicate that taccaoside may promote apoptosis and necrosis by activating caspase-3 and -8. The upregulation of Bax and downregulation of Bcl-2 were also observed in SMMC-7721 and Bel-7404 cells after treatment with taccaoside. Increased PARP cleavage was also observed in SMMC-7721 and Bel-7404 cells following taccaoside treatment (Figure 6).

Discussion

In this study, we showed that taccaoside, a steroidal saponin isolated from *Schizocapsa plantaginea* (Hance), induced cytotoxicity, apoptosis and cell cycle arrest in two HCC cell lines. Western blot results indicated that taccaoside induced apoptosis by modulating apoptosis-related proteins.

Taccaoside is a biologically active compound found in various types of traditional Chinese medicine, which represents another route to the discovery of therapeutic agents
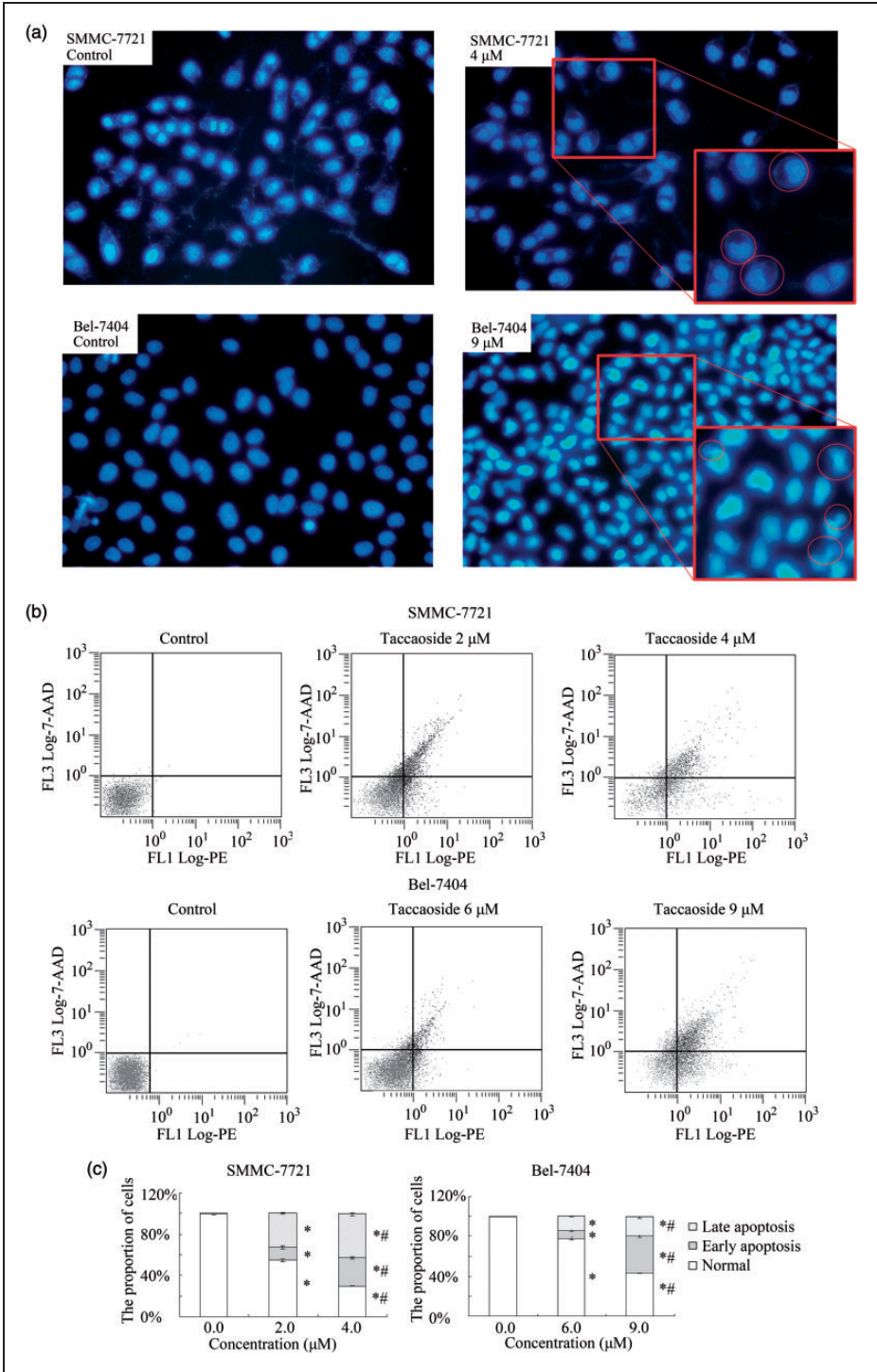
Figure 3. Taccaoside induced apoptosis in SMMC-7721 and Bel-7404 cells.
Figure 3(a). Effect of taccaoside on HCC cell morphology. SMMC-7721 cells were exposed to 4μM of taccaoside for 24μh and Bel-7404 cells were exposed to 9μM of taccaoside for 24μh. Apoptotic cells with
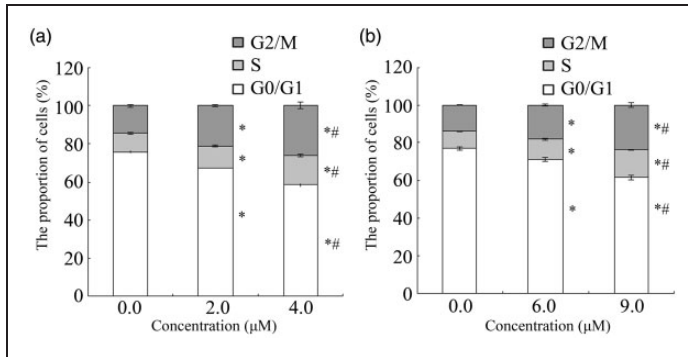
Figure 4. SMMC-7721 (a) and Bel-7404 cells (b) cell cycle after treatment with taccaoside. Data are presented as the mean ± SD (n = 3). *$P < 0.01$ compared with control group; #$P < 0.01$ compared with lowest dosage group.

for HCC. Although compounds from the *Taccaceae* family have been approved for cancer therapy in China,[2,3] their anticancer mechanism remains poorly understood. Determining the mechanism of action of taccaoside is therefore the key to understanding the treatment effects of *Taccaceae*.

In a previous study, taccaoside was found to have significant antiproliferation effects on cancer cell lines, inhibiting the HCC cell lines HepG2, Bel-7402 and SMMC-7721 with IC50 values of 1.2, 10.87 and 3.89 µM, respectively.[5,6] In another study, taccaoside had a significant antiproliferative effect in cancer cell lines including HEK293 and HL60.[11] Despite multiple reports documenting the cytotoxicity of taccaoside, the mechanism behind its cytotoxic effect remains unclear.

An imbalance between proliferation and apoptosis is a characteristic of HCC cells, an the antiapoptotic signal induced by multidrug resistance presents challenges in the

treatment of HCC using chemotherapy.[12] Regulation of the apoptosis signal in HCC cells is therefore a central focus in the treatment of HCC.

Several types of steroidal saponins can inhibit HCC cell proliferation by inducing apoptosis and blocking the cell cycle.[13,14] In the present study, taccaoside showed significant inhibition of two HCC cell lines, in line with the results of these previous studies. Based on our results, we show for the first time that taccaoside markedly increases the rate of apoptosis in SMMC-7721 and Bel-7404 cells as confirmed by the morphological changes observed. Furthermore, taccaoside may also inhibit HCC cell proliferation by blocking the cell cycle, as our results show that taccaoside decreased the percentage of cells in the G0/G1 phase by blocking cells in the G2/M or S phase.

Caspases are the primary death proteases, and their activation often triggers

Figure 3. Continued

nuclear fragmentation are enclosed within circles.

Figure 3(b). Apoptosis induced by taccaoside in HCC cell lines. SMMC-7721 cells were treated with 0, 2 and 4 µM of taccaoside for 24 h and Bel-7404 cells were treated with 0, 6 and 9 µM of taccaoside for 24 h.

Figure 3(c). The number of apoptotic cells is expressed as a percentage of the total cell number. The apoptotic rates of SMMC-7721 and Bel-7404 cells were markedly increased after exposed to taccaoside for 24 h. Data are presented as the mean ± SD (n = 3). *$P < 0.01$ compared with control group; #$P < 0.01$ compared with lowest dosage group.
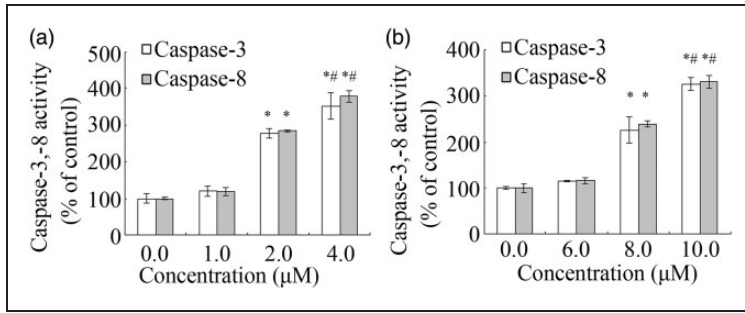
Figure 5. Taccaoside induced caspase-3 and -8 activation in SMMC-7721 and Bel-7404 cells. (a) SMMC-7721 cells were treated with 0, 1, 2 and 4 μM of taccaoside for 24 h. (b) Bel-7404 cells were treated with 6, 8 and 10 μM of taccaoside for 24 h. Data are presented as the mean ± SD ($n = 3$). *$P < 0.01$ compared with control group; #$P < 0.01$ compared with lowest dosage group.
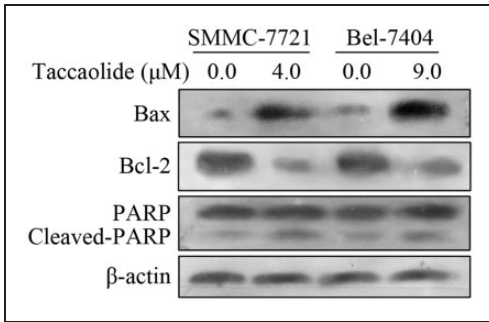

Figure 6. Taccaoside affected the expression of apoptosis-related proteins in SMMC-7721 and Bel-7404 cells. SMMC-7721 cells were treated with 4 μM of taccaoside for 24 h and Bel-7404 cells were treated with 9 μM of taccaoside for 24 h. β-actin was used as an internal control.

apoptosis and cell death. Among the caspase family, caspase-3 is the primary executioner of apoptotic death,[15] and morphological changes and caspase-8 activation[16] are necessary for caspase-3 activation. Using a caspase activity assay, we showed that caspase-3 and caspase-8 were activated following taccaoside treatment. Cell morphological changes observed by Hoechst 33258 staining also indicated caspase activation. Our results suggest that taccaoside promotes an anticancer effect in HCC cells via caspase-dependent apoptosis.

Our results also show that taccaoside regulates apoptosis-associated proteins in SMMC-7721 and Bel-7404 cells. Following exposure to taccaoside for 24 h, PARP cleavage, an important indicator of apoptosis,[17] was increased. Taccaoside also increased Bax expression and decreased Bcl-2 expression. The upregulation of Bcl-2 can lead to the blocking of various apoptotic signals,[18] while the upregulation of Bax leads to the activation of caspases.[19] The regulatory effect of taccaoside on Bax and Bcl-2 protein expression may play an important role in taccaoside-induced apoptosis.

In conclusion, we showed that taccaoside inhibited SMMC-7721 and Bel-7404 cell proliferation by inducing apoptosis and causing cell cycle arrest. Caspase-3 and -8 activation may also contribute to taccaoside-induced apoptotic cell death.

Declaration of conflicting interest

The Authors declare that there is no conflict of interest.

Funding

References

1. Ngok FK, Kel'ginbaev AN, Gorvits MB, et al. A new steroid glycoside, taccaoside, from Tacca cheancer. *Chem Nat Compd* 1980; 16: 262–264.

2. Huang XC. *The primary color profiles of Chinese anti-cancer herbals.* Nanning, Guangxi, China: Guangxi Science and Technology Press, 2002, pp.41–43.

3. Dai B. *Chinese modern Yao medicine.* Nanning, Guangxi, China: Guangxi Science and Technology Press, 2009, pp.405–408.

4. Jiang J, Yang H, Wang Y, et al. Phytochemical and pharmacological studies of the genus Tacca: a review. *Trop J Pharm Res* 2014; 13: 635–648.

5. Liu ZH, Yan H, Si YA, et al. Bioactive spirostane glycosides from Tacca plantaginea. *Steroids* 2016; 106: 86–92.

6. Wang YB, Zhang YC, Zhu ZY, et al. Exploration of the correlation between the structure, hemolytic activity, and cytotoxicity of steroid saponins. *Bioorg Med Chem* 2007; 15: 2528–2532.

7. Altekruse SF, McGlynn KA, Dickie LA, et al. Hepatocellular carcinoma confirmation, treatment, and survival in surveillance, epidemiology, and end results registries, 1992–2008. *Hepatology* 2012; 55: 476–482.

8. Maluccio M and Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin* 2012; 62: 394–399.

9. Wang S, Wu X, Tan M, et al. Fighting fire with fire: poisonous Chinese herbal medicine for cancer therapy. *J Ethnopharmacol* 2012; 140: 33–45.

10. Qiu FL and Zhou J. Two new steroidal saponins from TACCA plantaginea root. *Plant Diversity* 1985; 7: 1–3.

11. Liu H, Chou GX, Wang JM, et al. Steroidal saponins from the rhizomes of dioscorea bulbifera and their cytotoxic activity. *Planta Med* 2011; 77: 845–848.

12. Moreno-Càceres J and Fabregat I. Apoptosis in liver carcinogenesis and chemotherapy. *Hepat Oncol* 2015; 2: 381–397.

13. Long FY, Chen YS, Zhang L, et al. Pennogenyl saponins induce cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells. *J Ethnopharmacol* 2015; 162: 112–120.

14. Li YJ, Wang XR, Cheng SL, et al. Diosgenin induces G2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma cells. *Oncol Rep* 2015; 33: 693–698.

15. Brentnall M, Rodriguez-Menocal L, De Guevara RL, et al. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biol* 2013; 14: 32.

16. Janicke RU, Sprengart ML, Wati MR, et al. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998; 273: 9357–9360.

17. Soldani C and Scovassi AI. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis* 2002; 7: 321–328.

18. Kontos CK, Christodoulou ML and Scorilas A. Apoptosis-related BCL2-family members: key players in chemotherapy. *Anticancer Agents in Med Chem* 2014; 14: 353–374.

19. Finucane DM, Bossy-Wetze E, Waterhouse NJ, et al. Bax-induced caspase activation and apoptosis via cytochrome C release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem* 1999; 274: 2225–2233.