Original Article

In vitro and *in vivo* suppression of hepatocellular carcinoma by Amorphophallus konjac tuber through regulation of survivin and bax

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Background: Amorphophallus konjac tuber (AKT) is used in traditional Chinese medicine (TCM) to treat various types of cancer. Our study aims to evaluate the antitumor activity of AKT against hepatocellular carcinoma both *in vitro* and *in vivo*, as well as the underlying mechanisms.

Methods: AKT was extracted by aqua and organic solutions. The antitumor activity of the organic extract was tested in hepatocellular carcinoma cell lines Huh7 and H22, the latter was also used to establish mouse tumor model. Cell apoptotic rate was analyzed by flow cytometry. *In vivo* expression of apoptosis associated proteins survivin and bax were analyzed by immunohistochemistry (IHC).

Results: AKT exhibited antitumor activity against hepatocellular carcinoma cell lines. *In vivo* suppression of H22 was comparable with 5-fluorouracil (5-FU). AKT could induce apoptosis through up-regulation of bax and down-regulation of survivin.

Conclusions: Our data showed that AKT suppressed hepatocellular carcinoma, and the mechanism was associated with regulation of survivin and bax expression, providing support to the clinical use of AKT-based medication to achieve optimized outcome in hepatocellular carcinoma patients.

Keywords: Traditional Chinese medicine (TCM); Amorphophallus konjac; hepatocellular carcinoma; apoptosis

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Introduction

Natural compounds from herbs compose a significant part of first-line antitumor drugs, including vincristine, camptothecin and paclitaxel. Since common malignant tumors readily develop chemoresistance, there is high demand for novel antitumor compounds. Natural herbal medicine has become a promising source for novel discoveries, such as curcumin (1), berberine (2), Hedyotis diffusa (3), and decoctions containing undefined bioactive ingredients (4).

There are about 170 species of Amorphophallus worldwide (5), mainly distributed in tropical and subtropical Asia and are used as a food source as well as in traditional Chinese medicine (TCM). The tuber from a major species of Amorphophallus, Amorphophallus konjac [hereafter referred to as Amorphophallus konjac tuber (AKT)], locally produced in Zhejiang, China, has been used in anticancer therapy in TCM for decades (6). Our group has been investigating the efficacy of AKT as well as the underlying mechanism for over 10 years with both clinical practice and laboratory research (7). Recently, an Iranian group has also reported the antitumor effect of AKT (8,9). Thus AKT has become an acknowledged source of natural antitumor compounds. In this article, we show that extracts of AKT could inhibit hepatocellular carcinoma both *in vivo* and

in vitro by induction of cell apoptosis through survivin and bax pathways.

Methods

Herbal medicine and reagents

AKT was purchased from Zhejiang Lin-An Medical Herbs, Co. Ltd. (Batch no. 061208), China. 5-Fluorouracil (5-FU) was from Jinghua Pharmaceutical Group Co. Ltd., China. Other chemical reagents (grade AR) were from Sigma (USA), Amresco (USA) or Aladdin (China).

Preparation of AKT extracts and chemical analysis

Dried AKT was grounded to fine particles, soaked in 8× volume of 95% ethanol overnight, and extracted twice by reverse flow, 2 h each time. The ethanol collected was further extracted by 2× volume of ligarine for twice. The ligarine extract was recovered and concentrated under reduced pressure using a rotary evaporator. The ethanol residuum was extracted by dH_2O twice, 10× and 8× volume respectively, the extract was concentrated as well. We did one mega-extraction using 15 kg of AKT and have stored the extracts in 4 ℃ with desiccants.

Analysis of the composition of the organic extract of AKT

To analyze the rough chemical composition of the AKT extract by ethanol and ligarine, phosphomolybdic acid reaction, acetic anhydride-concentrated sulfuric acid, chloroform-concentrated sulfuric acid, and oil patch reactions were carried out.

Cell culture and cell viability assay

Huh7 and H22 cells were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China. The tumor cell suspension of H22 hepatocellular carcinoma (H22) was induced by C3HA mice, and after subcutaneous transplantation of KM mice, the tumor was transferred to the abdomen. Cells were cultured in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) at 37 ℃ with 5% CO_2. The cell viability was measured by a CCK8 kit (Lianke Bio, China) at OD 405 nm. For the viability assays, cells were seeded in 96-well plates, 1×10^4 cells per well. For short-term treatments, desired drugs, compounds and extracts were added to the wells immediately afterwards and cultured with the cells for 24 h. For long-term treatments, fresh medium with desired reagents were added every 24 h. Then we added 10 μL of CCK-8 solution and NSCLC cells were incubated for an additional 3 h. Cell viability is calculated from the OD values in comparison with the blank. Relative cell viability was calculated as a percentage of untreated controls. The inhibition rate was calculated by the following formula: inhibition rate (%) =100×(ODctrl-ODtreated)/ODctrl; Cell viability (%) = ODtreated/ODctrl ×100. Each treatment condition has been repeated 4 times with at least 3 repeated wells each time.

In vivo H22 tumor model

Use of animals was approved by the institutional ethical committee following the National Research Council Guide the Care and Use of Laboratory Animals. In our experiments, mouse hepatoma H22 cells were intraperitoneally injected in adult male mice (KM strain, 18–20 g, provided by Slac Laboratory Animal, Shanghai, China). The ethical approval number is ZSLL-2009-126. The mice produced tumor ascites after 6–8 days, which is the first generation; then we sacrificed the mice after extraction of ascites, dilution count, intraperitoneal injection of mice again in the abdominal cavity, 6–8 days after the generation of ascites, which is the second generation, at last we repeated the second step to get the third generation of asite cells. The tumor cells were collected by centrifugation and re-suspended in PBS with the concentration of 1×10^7/mL. Zero point two mL of suspension containing 2×10^6 cells was seeded to the auxiliary region of each mouse. The mice were randomly divided to 5 groups, 10 in each group. AKT was administered by intragastric gavage at the doses of 100, 200 and 400 mg/kg respectively (0.2 mL/10 g body weight) for 10 days constitutively. 5-FU was used as positive control and 0.9% saline was the negative control. Both the saline and the 5-FU were administered by intragastric gavage with the volume of 0.2 mL/10 g body weight. Weight of the mice were recorded daily prior to drug administration. The mice were sacrificed at the 11th day by cervical luxation.

Tumor tissues were collected from the auxiliary region and weighed. The inhibition rates by different treatments were calculated based on the weight of the tumor masses. Based on following formula: inhibition (%) =100×(Weightctrl-Weighttreated)/Weightctrl.

Flow cytometry analysis

Freshly isolated H22 cells from the tumor mass were collected following tissue sectioning and digestion by trypsin, and were subjected to flow cytometry by apoptosis analysis kit (Beckman Coulter, USA) based on the manufacturer's instructions. Flow cytometry analysis was performed immediately afterwards (FACSAriaTM, BD Biosciences, USA).

Western blottings

Huh7 cells after desired treatment was scraped, spun down, washed by PBS and resuspended in RIPA buffer with standard protease and phosphatase inhibitors. The lysate was spun at 1,000× g for 10 min and the protein levels from the supernatant were quantified by BCA method (Lianke Bio, China). Proteins from different treatment groups were subjected to SDS-PAGE and transferred to PVDF membranes, which were incubated by primary antibodies (anti-survivin or anti-bax, ZSGB-Bio, Beijing, China; anti-actin, Lianke Bio, China) followed by HRP-conjugated secondary antibodies (Lianke Bio, China). The membranes were developed with an enhanced chemoluminescence kit (Lianke Bio, China). One representative image from three repeated experiments was shown. Quantitative analysis of protein levels determined by band intensities was carried out by ImageJ (v1.48u).

Immunohistochemistry (IHC)

Tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4 mm sections, then subjected to hematoxylin eosin (HE) staining or IHC. For IHC, tissue sections were incubated with anti-survivin or anti-bax antibodies (both from ZSGB-Bio, Beijing, China). The positive signals were amplified by a biotinylated peroxidase-conjugated streptavidin system (Bio-Genex Laboratories, USA). All specimens were observed and photographed (BX51T-32H01, Olympus, Japan).

Statistical analysis

Each *in vitro* experimental condition was performed at least 3 times with multiple repeats, and the data were analyzed by SPSS (v13.0). The statistical significance was calculated by one-way ANOVA to compare multiple groups, or by student's *t*-test when only two groups were compared. $P<0.05$ was considered significant, $P<0.01$ was considered highly significant.

Results

The chemical composition of AKT extracts

After primary separation, we have obtained different of AKT extracts with the following yields calculated from the dry weights: auqa-based extract 223.1% and organic solvent-based extract 5.73%, which constituted a small proportion to the dry weight. By simple chemical reaction analysis, the organic extract was positive in oil patch reaction indicating the presence of volatile oil, and negative for grease, steroids and triterpene.

The in vitro antitumor activity AKT extracts

We have tested the antitumor effect of aqueous and organic extracts of AKT in hepatocellular carcinoma cell lines Huh7 (human) and H22 (mice) (*Figure 1A,B*). The aqueous extract had very little inhibitory effect, while the organic AKT extract exhibited a strong antitumor effect with IC50 of 35–45 μg/mL based on the inhibition curve (*Figure 1A,B*). Furthermore, consecutive treatment of 50 μg/mL AKT annihilated Huh7 and H22 within 7 days (*Figure 1C,D*). In long-term treatments, lower dose of AKT (30 μg/mL) had similar potency as the higher dose (50 μg/mL) to totally inhibit the tumor cell viability within 7 days.

The in vivo antitumor activity of AKT

The organic extract of AKT was used for the *in vivo* studies. For the *in vivo* and mechanistic studies, AKT were used considering its relatively high antitumor potency and potentially simpler chemical composition. In *Figure 2A,B*, after 10 days of continuous AKT treatment by intragastric gavage at 100 mg/kg in KM mice, the hepatocellular carcinoma H22 tumor mass was significantly decreased. HE staining showed that the tumor cells were diffusely distributed with different size, irregular shape, the proportion of nuclear cytoplasm was increased, more common mitosis, interstitial vascular dilatation and congestion, large necrosis in the tumor tissue and lymphocytic infiltration, but there was no significantly difference between control and AKT-treated tissues (*Figure 2C*). The inhibition rate was 32.4%, comparable with 25 mg/kg 5-FU treatment (35.4%) (*Figure 2D*). Increase of AKT dose to 200 and 400 mg/kg

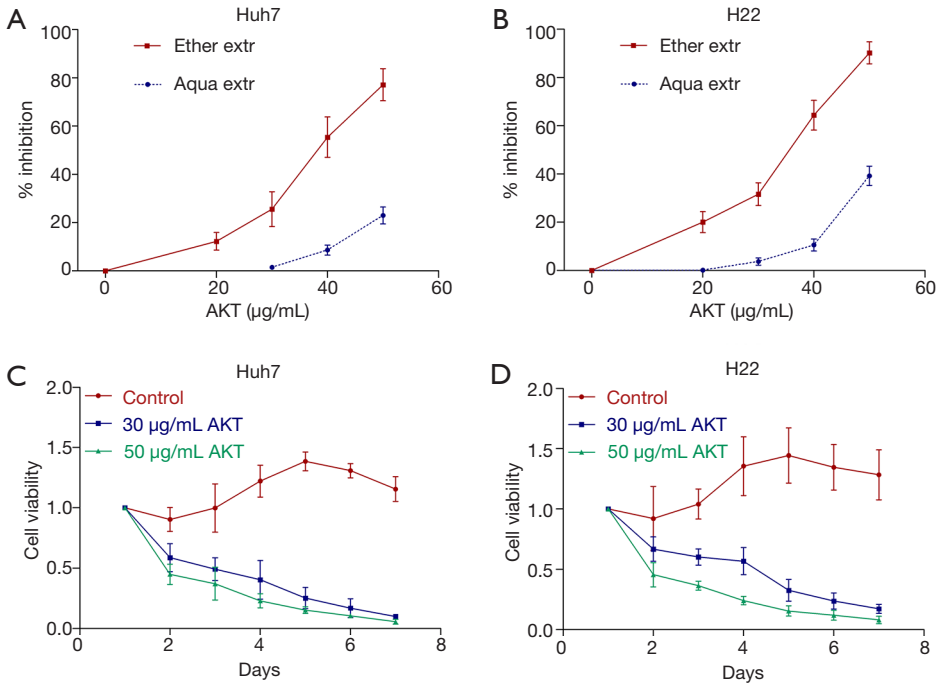

Figure 1 The *in vitro* antitumor activity of AKT. (A,B) The suppression of Huh7 and H22 cells by the aqueous and organic extracts of AKT; (C,D) AKT were added to cultured Huh7 and H22 tumor cells for 7 days, with desired doses added fresh daily. The inhibition rates were calculated after cell viability assays and were presented as mean ± SE (n=4). AKT, Amorphophallus konjac tuber.

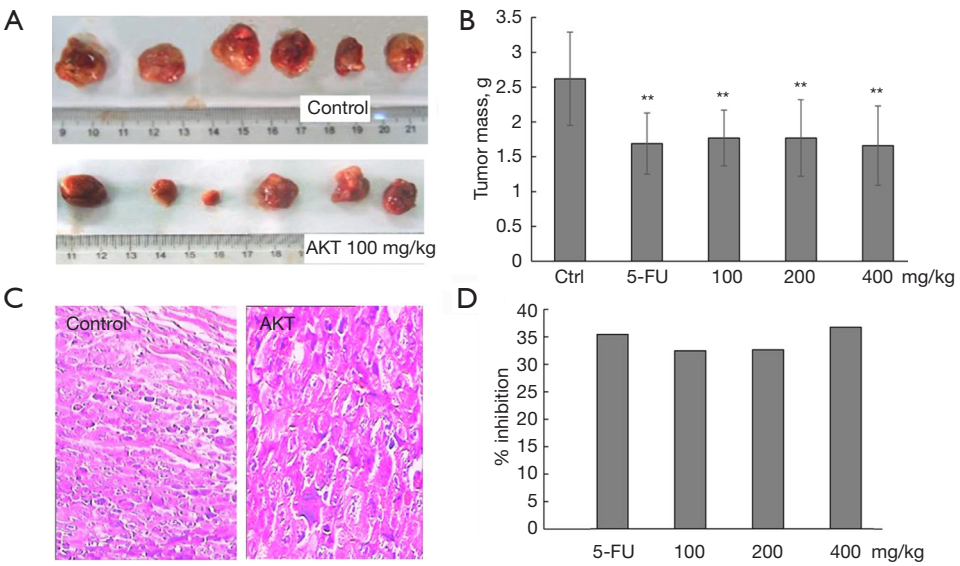

Figure 2 The *in vivo* antitumor activity of AKT. H22 tumor models were established in KM mice and treated by saline (control), AKT at 100, 200 and 400 mg/kg, and 5-FU at 25 mg/kg. Tumor masses were measured upon sacrifice of mice after desired treatments. (A) Gross observation of sectioned tumor tissues; (B) comparison of tumor mass. Data were presented as mean ± SD from 10 mice in each group; (C) HE staining of tissue sections from the control group (200×) and from the AKT-treated group (400×); (D) the inhibition rates of each AKT-treated group calculated based on the mean values of the tumor mass presented in C. **, P<0.01, compared with the control group; 5-FU, 5-fluorouracil; AKT, Amorphophallus konjac tuber.

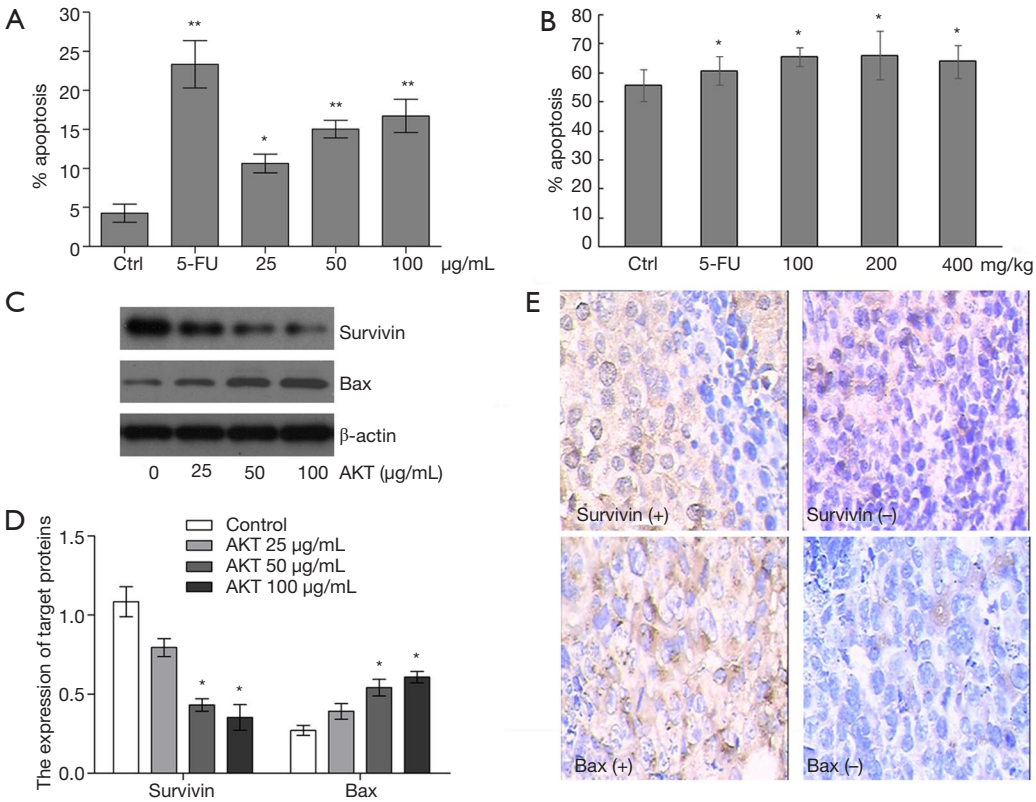

Figure 3 AKT-induced apoptosis by regulation of survivin and bax levels. (A) Apoptotic rates of Huh7 cells analyzed by flow cytometry; (B) the apoptotic rates of isolated tumor cells from H22 mice model analyzed by flow cytometry. Data were presented as mean ± SD, n=5; (C) Western blotting analysis of survivin and bax in Huh7 cells following AKT treatment; (D) statistical analysis of ImageJ-based densitometry of survivin and bax normalized by actin; (E) representative images of immunohistochemistry (IHC) analysis of survivin and bax in H22 tumor tissues grown in mice and treated by AKT. All images were 400×. *, P<0.05; **, P<0.01. 5-FU, 5-fluorouracil; AKT, Amorphophallus konjac tuber.

did not further enhance the inhibition rates. There was no significant difference between the 5-FU group and the AKT groups at different doses.

AKT could induce cell apoptosis.

We analyzed the change of apoptotic rate in AKT-treated Huh7 cells. In *Figure 3A*, flow cytometry was carried out to detect the apoptotic cells induced by AKT after co-staining of annexin V-FITC and PI. After treatment of 24 h, 5-FU induced significant increase of apoptosis; 25 μg/mL AKT did not induce significant increase of apoptotic cells. After cells were treated by 50 and 100 μg/mL AKT, cell apoptosis was significantly increased. Apoptotic rates analyzed by morphological observation and flow cytometry were comparable.

We also analyzed the apoptotic rate in freshly isolated H22 tumor cells grown *in vivo* by flow cytometry (co-staining of annexin V-FITC and PI). From *Figure 3B*, the apoptotic rate of isolated H22 tumor cells from the control group was 55.75%, increased to 65.53% after mice were treated by 5-FU (25 mg/kg, intragastric gavage, 10 days), and to 60–66% after treatments of AKT (100, 200 and 400 mg/kg). Increase of AKT from 100 to 400 mg/kg did not further increase the apoptotic rate. Bax and survivin were detected by Western blotting (*Figure 3C,D*) and IHC (*Figure 3E*). In *Figure 3C*, after AKT treatment, levels of survivin had decreased and levels of bax had increased. In *Figure 3D*, by statistical analysis of repeated Western blottings based on ImageJ, in the AKT 50 and 100 μg/mL groups, levels of survivin was significantly decreased compared with the control group, and levels of bax was significantly increased. In the AKT 25 μg/mL group, there was a trend of decrease of survivin and increase of bax

Table 1 Expression of apoptosis-regulatory proteins in hepatocellular carcinoma grown in mice

Group	n	Survivin+, n	Survivin–, n	Survivin+, %	Bax+, n	Bax–, n	Bax+, %
Control	10	9	1	90	2	8	20
5-FU	9	3	6	33.3**	7	2	77.8**
AKT	26	10	16	38.5*	22	4	84.6*

KM mice with established H22 tumor received saline (control), 5-FU and AKT. Expression levels of survivin and bax proteins were determined by IHC. *, P<0.05; **, P<0.01, compared with the control group. 5-FU, 5-fluorouracil; AKT, Amorphophallus konjac tuber; IHC, immunohistochemistry.

levels, although no statistical significance was revealed. Representative IHC positive and negative images from the AKT treatment group are shown in *Figure 3E*. The semi-quantitative data is presented in *Table 1*. In H22 tumors without treatment, 90% samples were survivin positive and 20% samples were bax positive. After treatment by AKT (100, 200 and 400 mg/kg dosage groups combined), the survivin positive rate dropped to 38.5% and the bax positive rate increased to 84.6%. The changes were comparable with the 5-FU group.

Discussion

In 2014, Ansil *et al.* used similar extraction method from the tuber of Amorphophallus campanulatus, which this the same species of Amorphophallus konjac, and found antitumor activity of the extract against N-nitrosodiethylamine-induced hepatocellular carcinoma in rats (8) and against human hepatoma cell lines (9). We found the ligarine-based extraction produced potent antitumor extract, while the aquatic extract did not have much effect, suggesting ethanol and ligarine based extraction methods to be more appropriate for the purpose of antitumor treatments of Amorphophallus konjac. From our data, the ethanol-ligarine extract of AKT contained various organic compounds including nitrogen and sulfur-containing compounds. Further separation and identification of these components are to be carried out in future studies by nuclear magnetic resonance (NMR) etc. The bioactive chemical is to be identified. However, according to the holistic theory of TCM, it is also likely that the efficacy is due to the combinational use of multiple ingredients.

A TCM decoction contains hundreds of different chemical components and modulate many molecular targets simultaneously to result in complicated cascades of cellular events. Qing-Yi-Hua-Ji formula, a decoction with AKT as one of the major ingredients, could increase the survival of late stage pancreatic cancer patients (10), and has been reported to affect multiple downstream targets including Ski (11), Notch-4 and Jagged-1 (12). We found AKT could induce apoptosis both *in vitro* and *in vivo* and regulated the levels of survivin and bax both *in vitro* and *in vivo*. Recent studies have been reported that Amorphophallus tuber extract in inhibits the activity of cytotoxicity and promoted apoptosis in human colon carcinoma cells and hepatoma cells (9,13). Survivin belongs to the family of inhibitors of apoptosis proteins (IAP) that regulate cell death by inhibition of caspase activation. It is expressed only in cells in the G2/M phase, but absent in differentiated cells (14,15). Survivin is highly expressed in most human tumors and fetal tissues, and has thus become a biomarker and a therapeutic target in cancer (16). From our data, AKT could decrease the levels of the anti-apoptotic proteins survivin and simultaneously increase the levels of the pro-apoptotic proteins bax. AKT could regulate apoptosis through the IAP family of proteins, although was not necessarily dependent on them.

Amorphophallus konjac is well tolerated as a food source in some countries. Among the 94 organic compounds identified from konjac powder, none was toxic by the standards of USFDA (17). It contains glucomannans, which are potentially applicable in clinical use against obesity and diabetes. They can decrease body weight through regulating food absorption in the gut, and have been registered in clinical trials in adults (18) and in children (18,19). The tuber part of Amorphophallus konjac of has been used in cancer treatment in TCM for decades. Glucomannans, the most studied bioactive component from konjac powder, are typically extracted by water followed by coagulation by ethanol, which produces much higher yields compared with the organic solvents-based isolation methods (20). Our data and previous report indicate that the potent fractions with antitumor activity from AKT are contained in the ethanol-ligarine extract. Although the inhibition was dose-dependents in the 0–50 μg/mL range at the 24 h time-point, the inhibition reached saturation at low doses when

using in long-term treatment. From the *in vivo* experiments, increase of AKT from 100 to 400 mg/kg also resulted in similar inhibition rates. Although safe to consume, our data suggest that AKT-based medication in relatively smaller doses would be efficient to achieve desired effects.

Overall, our findings strongly support the feasibility to isolate novel antitumor compounds from the Amorphophallus konjac, which could be used as an alternative or in combinational regimen in the clinic against hepatocellular carcinoma.

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.01.26). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Use of animals was approved by the institutional ethical committee following the National Research Council Guide the Care and Use of Laboratory Animals. The ethical approval number is ZSLL-2009-126.

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