CYTOTOXICITY OF ACULEATISIDE-A TO HEPATOCELLULAR CARCINOMA CELLS DETERMINED BY MTT BIOASSAY

Alaa Mohamed Nafady[1*] and Omar Mahmoud Mohamed Mohafez[2]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Assiut 71524, Egypt*
[2]*Department of Biochemistry, Faculty of Pharmacy, Al-Azhar University, Assiut 71524, Egypt*

من خلاصة الميثانول للثمار الطازجة لنبات السولانوم ريفليكسوم تم فصل مركب اساسى وقد تم التعرف عليه كالتالى: اكيولياتيزيد ا ١ وقد تم التعرف على المركب عن طريق الدراسات الطيفية المختلفة لمطياف الرنين النووى المغناطيسى البروتونى والكربونى وكذلك بالمقارنة بالابحاث المنشورة. وقد تمت دراسة التاثير السمى للمركب على خلايا الكبد السرطانيه وكذلك الية عمله ولقد تم استنتاج ان المركب يؤدى الى تاثير ملحوظ فى تثبيط نمو خلايا الكبد السرطانيه (HepG-2) عند احداث الموت المبرمج للخلايا ولقد شوهدت تغيرات واضحة للصفات العيانية الخار. . هذه النتائج ولاول مرة اوضحت ان المركب له القدرة على الحد من انتشار الخلايا الكبدية السرطانيه وذلك فى التجارب المعملية بالاضافة الى تنظيم تراجعى لمعايير السرفيفين و2-Bcl وكذلك تنظيم تقدمى من Bax و P[53] والتى يمكن ان تكون احد اهم اليات تدمير الخلايا السرطانية.

*From the methanolic extract of fresh fruits of Solanum reflexum Schrank, a major compound was isolated and identified as; aculeatiside-A **1**. The structure of the isolated compound was elucidated on the basis of ^1H and ^{13}C and DEPT-^{13}C-NMR; in addition to comparison with reported data. The cytotoxic effect of aculeatiside-A on hepatocellular carcinoma cells and its mechanism was investigated. It was accomplished that aculeatiside-A inhibit the growth of HepG-2 cells significantly by induction of apoptosis. Marked morphological changes of apoptosis were observed clearly. The results for the first time demonstrate that aculeatiside-A has significant anti-proliferation effect by induction of apoptosis on hepatocellular carcinoma cells in-vitro, down regulation of Survivin and Bcl-2 expressions as well as up regulation of Bax and P^{53} expression may be one of the important apoptotic inducing mechanisms.*

INTRODUCTION

Plants are inexhaustible sources of complex chemical molecules produced as a result of their secondary metabolism. Mainly, alkaloids and saponins have attracted special interest owing to its vital and therapeutic effect on the human body. *Solanum* is the largest genus in Solanaceae family with about 1700-2000 species distributed throughout the temperate and tropical regions of the world[1]. *Solanum reflexum* Schrank has been used to treat toothache, skin complaints, bronchitis and rheumatism in China and solasodine, solasonine and solamargine are known as constituents of this plant[2]. In our studies on the oriental *Solanum* plants, we have now isolated compound **1**[3&4], which is a steroidal glycoside,

named aculeatiside-A for the first time from the fruits of *Solanum reflexum* Schrank, and elucidated its structure. No records were observed regarding the cytotoxicity bioassay of aculeatiside-A upon tracing its biological activities, which encouraged us to investigate its cytotoxic action.

Despite recent advance in understanding the molecular biology of hepatocellular carcinoma cells and the induction of some new chemotherapeutic agents for the treatment of this malignant disease, there are few efficient therapeutic measures or regimes especially for the patients who are in the mid or final stages, and the dismal 5-year survival rate has not changed too much for this leading cause of cancer deaths in the world[5&6]. Therefore, it is a permanent subject to find new drugs and

*Corresponding author: Alaa Mohamed Nafady, E-mail: alaa12nafady@yahoo.com

effective therapies for the clinical treatment of hepatocellular carcinoma. It is recently reported that plant-derived compounds are known to have curative potential.

The present study was undertaken to investigate the cytotoxic effect of aculeatiside-A on hepatocellular carcinoma cells and its possible mechanism. Hepatocellular carcinoma cell line, HepG-2 cells, was used. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT). Cell morphology was observed by the phase contrast microscope under normal or trypan blue stain. Reverse transcriptase-polymerase chain reaction was used to estimate expression of Survivin and P^{53} mRNAs while Western blot analysis was used to detect Bax and Bcl-2 expressions.

EXPERIMENTAL

NMR spectra (^{1}H, ^{13}C, DEPT-^{13}C-NMR) were measured in pyridine-d_5 on a JEOL α-500 spectrometer (Japan). Column chromatography was carried out on silica gel 60 (70-230, 230-400 mesh, Merck). TLC was carried on precoated silica gel plates G60 F_{254} (0.2 mm Aluminium sheets, Merck) and RP-18 F_{254S} (0.2 mm Aluminium sheets, Merck). Plates were visualized by spraying with 20% v/v H_2SO_4 in EtOH, allowed to dry at room temperature and heated at 110-140°C for 1-2 min. All solvents were double distilled prior using. All chemicals are of analytical grade. Authentic sugar samples were obtained from Sigma-Aldrich Company (Germany).

TLC solvent system
$CHCl_3$-MeOH (98:2, 95:5, 90:10, 85:15)
$CHCl_3$-MeOH-H_2O (9:1:0.1, 9:1:0.2, 8:2:0.1, 8:2:0.2, 7:3:0.1, 7:3:0.2, 7:3:0.5, 65:35:0.1).

Chemicals for cytotoxicity bioassay
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). RNA extraction kit from (BioFlux, Bioer Technology Co., Ltd.). RT using RevertAid^{TM} First strand cDNA synthesis kit (Fermentas). PCR using Tag master/high yield (Jena Bioscience). DNA ladder using low range DNA ladder 50-1kbp linear scale (Jena Bioscience). Protein marker using page Ruler^{TM} prestained protein ladder

(Fermentas). PVDF membrane (Amersham Hybond^{TM}-P GE Healthcare) were used.

Plant material
Fresh fruits of *Solanum reflexum* Schrank were collected from the botanical garden of Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamto, Japan. The plant was authenticated by Dr. Tatemi Yoshida, National Research Institute of Vegetables, Ornamental Plants and Tea, Ministry of Agriculture, Forestry and Fisheries, Ano, Mie, Japan. A voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Assiut, Egypt.

Extraction and fractionation
The fresh fruits of *Solanum refelxum* (2 Kg) was cut into small pieces and extracted three times with hot methanol (5 L. each time). The methanolic extracts were combined and then concentrated under reduced pressure to give 30 g of yellowish brown residue, labeled SRF, which showed the presence of one major spot, in addition to five minors on TLC. Ten grams of SRF fraction were dissolved in $CHCl_3$-MeOH-H_2O (8:2:0.2), chromatographed on silica gel C.C. (70-230 mesh, 80×4cm) as a stationary phase. Elution was performed using $CHCl_3$-MeOH-H_2O (8:2:0.2) to (6:4:1) in a manner of increasing polarity. The effluents were collected in fractions (10 ml each). Similar fractions were grouped together and concentrated under reduced pressure. Fractions 10-15 eluted with $CHCl_3$-MeOH-H_2O (9:1:0.1) were pooled together, the solvent was distilled off to afford a dry residue (1.2 g), labeled group SRF-2. Further silica gel C.C. Using $CHCl_3$-MeOH-H_2O (8:2:0.2) to (7:3:0.2) afforded granular white powder labeled compound **1** (100 mg), which showed a clear single spot on TLC using system $CHCl_3$-MeOH-H_2O (8:2:0.5). The compound was precipitated from methanol by addition of diethyl ether and subjected to acid hydrolysis in addition to spectral investigations.

Analysis of sugar component
Compound **1** (10 mg) was hydrolysed with 2 mol/L HCl-MeOH at 80°C for 3 hrs. The reaction mixture was neutralized with 2 mol/L NaOH in H_2O and extracted with $CHCl_3$,

concentrated under reduced pressure and checked by TLC [precoated silica gel, *n*-BuOH–C₅H₅N–H₂O (8:4:3)] alongside with authentic sugar samples, which indicated the existence of glucose and rhamnose moieties.

Compound 1: White granular powder, (100 mg), ^{1}H-NMR (Pyridine-d_5), δ_H 0.83 (3H, s, H₃-18), 1.04 (3H, s, H₃-19), 1.09 (3H, d, *J*= 6.71 Hz, H₃-21), 1.39 (3H, s, H₃-27), 1.62 (3H, d, *J*= 5.5 Hz, H₃-6′′′′), 1.75 (3H, d, *J*= 6.11 Hz, H₃-6′′), 3.90 (1H, d, *J*= 9.76 Hz, H-26$_b$), 4.10 (1H, d, *J*= 10.36 Hz, H-26$_a$), 4.94 (1H, d, *J*= 7.33 Hz, H-1′), 5.3 (1H, partially obscured by C₅D₅N signal, H-1′′′′), 5.81 (1H, s, H-1′′), 6.34 (1H, s, H-1′′′). ^{13}C-NMR (Pyridine-d_5, 125 MHz) was illustrated on table 1.

Table 1: ^{13}C–NMR (125 MHz, pyridine-d_5) data of compound **1**.

Carbon atom	(δ)	DEPT	Carbon atom	(δ)	DEPT
C-1	37.52	CH₂	C-1′	100.29	CH
C-2	30.16	CH₂	C-2′	78.15	CH
C-3	78.38	CH	C-3′	78.38	CH
C-4	39.85	CH₂	C-4′	77.93	CH
C-5	140.79	C	C-5′	76.87	CH
C-6	121.82	CH	C-6′	61.34	CH₂
C-7	32.27	CH₂	*Rha*		
C-8	31.65	CH	C-1′′	102.91	CH
C-9	50.28	CH	C-2′′	72.52	CH
C-10	37.13	C	C-3′′	72.70	CH
C-11	21.10	CH₂	C-4′′	73.88	CH
C-12	38.98	CH₂	C-5′′	69.49	CH
C-13	40.51	C	C-6′′	18.62	CH₃
C-14	56.47	CH	*Rha′*		
C-15	32.27	CH₂	C-1′′′	102.02	CH
C-16	80.98	CH	C-2′′′	72.52	CH
C-17	62.45	CH	C-3′′′	72.70	CH
C-18	16.19	CH₃	C-4′′′	73.88	CH
C-19	19.40	CH₃	C-5′′′	70.44	CH
C-20	38.58	CH	C-6′′′	18.47	CH₃
C-21	15.15	CH₃	*Glc′*		
C-22	120.27	C	C-1′′′′	105.39	CH
C-23	33.10	CH₂	C-2′′′′	75.35	CH
C-24	33.91	CH₂	C-3′′′′	78.38	CH
C-25	83.88	C	C-4′′′′	71.65	CH
C-26	77.43	CH₂	C-5′′′′	78.15	CH
C-27	24.37	CH₃	C-6′′′′	62.72	CH₂
Glc					

Cell culture

HepG-2 cells were purchased from Serum and Vaccine Research Institute, Cairo, Egypt. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FBS), containing 100 U/ml penicillin and 100 µg/ml streptomycin, in a humidified incubator with 5% CO₂ at 37°C. Cells were passed twice weekly and routine examination was also done for mycoplasma contamination. Cells in logarithmic growth phase were used for the experiments[7].

Cell viability assay

The viability of the cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, HepG-2 cells in logarithmic growth phase were collected, and 5000 cells/well were dispensed within 96-well culture plates in 100µl volumes. Then different concentrations of aculeatiside-A (25, 50, 100 and 200 µg/ml final concentration) were added in different wells. We added 1% DMSO, solvent of the aculeatiside-A, to the control wells. Every concentration or the control was contained in three parallel wells. Culture plates containing the aculeatiside-A were then incubated for 24, 48 and 72 hrs prior to the addition of tetrazolium reagent. MTT working solution, 5 mg MTT/ml PBS, was sterilized by filtration using 0.45 µm filters. Each well received 20 µL of MTT working solution and incubated for further 5 hrs. The culture medium was removed carefully without disturbing the formazan layer. The water insoluble formazan was dissolved by addition of 100 µl DMSO to each well. Amount of formazan was determined by measuring the absorbance at 600 nm using, metertech 960, ELISA reader[8].

Detection of mRNA expressions using RT-PCR

The expressions of survivin and P^{53} in HepG-2 cells were examined by RT-PCR before and after the cells were treated with different concentrations of aculeatiside-A for

24, 48 and 72 hrs. The total RNA was extracted by using total RNA extraction kit from (BioFlux, Bioer Technology Co., Ltd.) according to the instruction manual. The first strand cDNA was synthesized according to the instruction manual of RevertAid[TM] First strand cDNA synthesis kit (Fermentas). The PCR performed using Tag master/high yield (Jena Bioscience) as following condition; pre-denaturing for 5 min at 94°C then denaturing at 94°C for 30 sec, annealing 55°C, and extension at 72°C for one min. The amplification was carried out in 28 cycle using (Biometra cycler). The PCR products were then run on 1.5% agarose gel in TAE buffer for 40 min, at 75V and then visualized by ethidium bomide staining.

Western blot analysis

For Western blotting, cells were washed with ice-cold PBS twice and lysed for 30 min at 4°C in NED buffer, then debris was removed by centrifugation for 15 min at 14,000 rpm at 4°C. 50 µg from each concentration were denatured by boiling for 5 min in 2% SDS and 5% 2-mercaptoethanol and loaded in each lane. SDS–PAGE was done at 100 volts for 2 hrs using 12% gel. The electrotransfer was done using T-77 ECL semi-dry transfer unit (Amersham Biosciences) for 2 hrs. The membranes were blocked in TBS buffer that contains 0.05 Tween and 5% non-fat milk for one hour. The membranes were reacted firstly with rabbit polyclonal antibodies; anti-Bcl-2 and anti-Bax at a dilution of 1:300 for 2 hrs, followed by extensive washes with PBS twice and TBST twice. Membranes then incubated with the secondary antibody, polyclonal goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma–Aldrich, Schelldorf, Germany), diluted 1:5000[9].

Oligonucleotides used for amplifications

NCBI reference sequence: NM_000546.4, NM_001168.2 and NM_002046.3 were used for design primers for P[53], survivin and GAPDH respectively. The coding sequences were used to design the primer pairs as shown in table 2.

Statistical analysis

All experiments were performed in triplicates and the results were expressed as mean ± SD. Statistical analysis was performed with t-test using SPSS-10 software.

Table 2: PCR primers used in this study.

P[53] primers (537bp)	Sense primer:	5- GAAGACCCAGGTCCAGATG-3
	Anti-sense primer:	5-GTAGTGGATGGTGGTACAGT-3
Survivin primers (558bp)	Sense primer:	5-CCGCATCTCTACATTCAAGA-3
	Anti-sense primer:	5-GCACCTCTGGTGCCACTTT-3
GAPDH primers (499bp)	Sense primer:	5-GATTTGGTCGTATTGGGCG-3
	Anti-sense primer:	5-GTCATGAGTCCTTCCACGA-3

RESULTS AND DISCUSSION

The ^{1}H-NMR spectra of compound **1** exhibited a set of signals due to three tertiary methyl groups at δ_H 0.83, 1.04 and 1.39 each (3H, s) and three secondary methyl signals at δ_H 1.09 (3H, d, J= 6.71 Hz), 1.62 (3H, d, J= 5.5 Hz) and 1.75 (3H, d, J= 6.11 Hz), in addition to four anomeric protons at δ_H 4.94 (1H, d, J= 7.33 Hz), 5.3 (1H, partially obscured by C_5D_5N signal), 5.81(1H, s) and 6.34 (1H, s)[3&4]. The afore mentioned data in combination with ^{13}C-NMR illustrated on table 1 suggested that compound **1** is a nautigenin tetraglycoside (Fig. 1); in which, its sugar component consists of one mole of glucose linked to C-26 together with an additional glucose unit and two moles of rhamnose linked to C-3 of the aglycone part[3&4]. The ^{13}C-NMR revealed the presence of three anomeric carbons at δ_C 100.29, 102.02 and 102.91, among which two anomeric carbon signals at δ_C 102.02 and 102.91 were ascribable to two terminal rhamnosyl moieties attached to C-2 and C-4 of an inner glucosyl molecule with anomeric carbon appeared at δ_C 100.29 and this was obvious from the down field shift of C-2 and C-4 from δ_C 75.3 and 71.6 to δ_C 78.15 and 77.93 respectively[3&4]. These data were assigned to β-chacotriosyl moiety attached to C-3 of the aglycone part. More over an extra glucose unit with anomeric carbon appeared at δ_C 105.39, could be attached to the C-26 of the aglycone moiety[3]. From the above mentioned data and by comparison with literature[9&10]; it was concluded that compound **1** deduced as: 3-O-[α-L-rhamnopyranosyl-(1→2)-α-L-rhamno-pyranosyl(1→4)-β-D-glucopyranosyl]nauta-genin-26-O-β-D-glucopyranoside, which is also known as aculeatiside-A[9&10]. This is the first reported occurrence of compound **1** in *Solanum reflexum* Schrank fruits.

Cell viability

To investigate the growth inhibition effect of aculeatiside-A on HepG-2 cells, we treated the cells with different concentrations of aculeatiside-A for 24, 48 and 72 hrs and measured the viability using MTT assay method. As shown in figure 2, aculeatiside-A had significant growth inhibition effect in a time and dose dependant manner. The percent of inhibition of cell proliferation (% IC) was 14.46, 32.35, 46.18 and 69.78 for 25, 50, 100 and 200 µg/ml after 24 hrs respectively. While it was 23.68, 43.73, 68.11 and 75.66 after 48 hrs respectively. Finally, the (% IC) was 33.72, 56.99, 78.77 and 89.71 after 72 hrs respectively.

To investigate the synergistic affect of curcumin, a known compound that inhibits growth of HepG-2 cells was chosen. We treated the cells with 10 µg/ml curcumin in addition to the aculeatiside-A for 24 hrs. Addition of curcumin causes inhibition of cells growth by 2.34, 1.84, 1.83 and 1.29 folds in comparison to that of aculeatiside-A alone as shown in figure 3.

Detection of cell apoptosis

To show whether the growth inhibition induced by aculeatiside-A in HepG-2 cells were caused by induction of apoptosis, cells were stained by trypan blue as shown in figure 4. The living cells attached to the matrix and spindle in shape but after the aculeatiside-A treatment, the cells detached and become spherical in shape. After treatment by 100 µg/ml aculeatiside-A for 72 hrs, the cells were collected and treated with 1% trypan blue. Most of cells showed the criteria of apoptosis that are condensation, small size and stained with deep blue color. The living cells are large and shining.

Expression of apoptosis related genes

After treatment with 100 µg/ml aculeatiside-A for 24, 48, and 72 hrs, RT-PCR analyses was used to detect the variation of apoptosis related genes. The results of RT-PCR demonstrate that the mRNA expression of P[53], apopototic gene, was up-regulated while the survivin, anti-apoptotic gene was down regulated (as shown in figure 5).

Expression of apoptosis related proteins

The cell were collected after treatment with 100µg/ml aculeatiside-A for 24, 48, and 72 hrs, washed and homogenized. Western blot analysis revealed over expression of Bax protein and it was time dependant, concomitantly with decrease in the expression of Bcl-2 protein.

In this study, we found that aculeatiside-A, could inhibit the proliferation by inducing apoptosis on hepatocellular carcinoma cells remarkably when the cells treated with
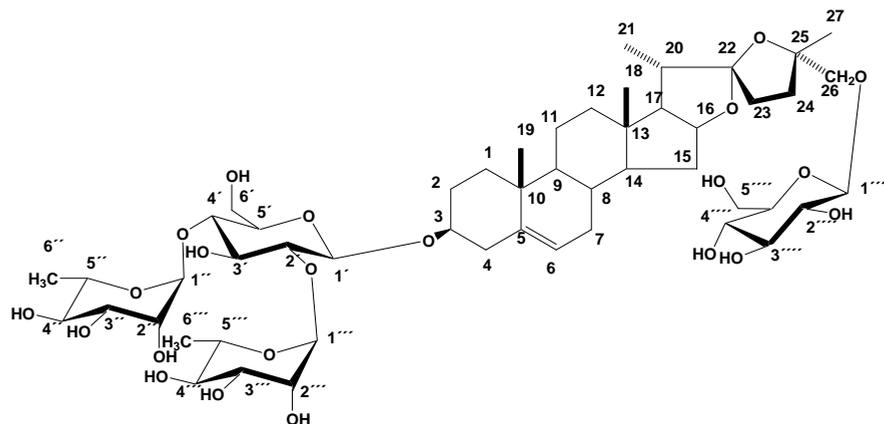
Fig. 1: Structure of Aculeatiside-A.
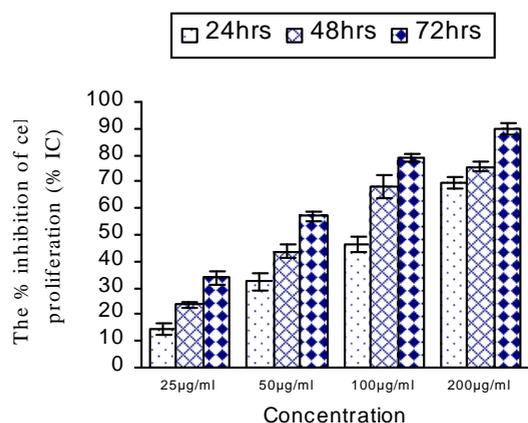
Fig. 2: Growth-inhibitory effects of aculeatiside-A on HepG-2 cells. Cells were treated with 25-200 µg/ml of Aculeatiside-A for 24, 48 or 72 hrs. Culture medium containing 1% DMSO was used as control. Cell viability was measured by the MTT assay. Values represent means ± SD of three independent experiments performed in triplicate.
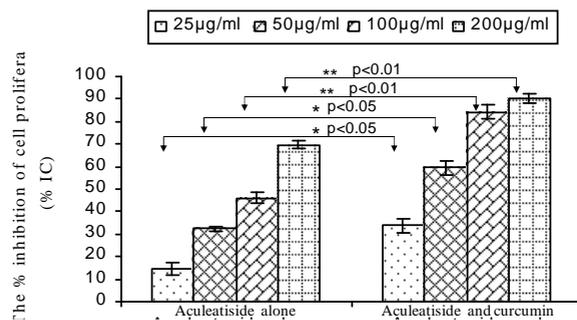
Fig. 3: Synergistic effect of curcumin on the growth-inhibitory effects of Acleotazide. Cells were treated with 25-200 µg/ml of aculeatiside-A combined with 10 µg/ml of curcumin for 24hrs. Cell viability was measured by the MTT assay. Values represent means ± SD of three independent experiments performed in triplicate.
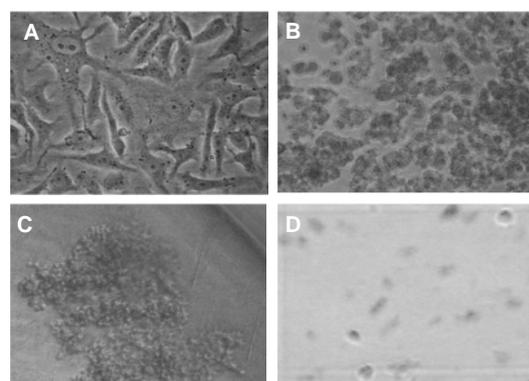
Fig. 4: Morphological changes of HepG-2 cells on exposure to acleotazide. A) Control cells B) and C) Cells treated with 100 µg/ml of aculeatiside-A for 24 and 48 hrs respectively. D) Cells stained with trypan blue after treating with 100 µg/ml of aculeatiside-A for 72 hrs. Arrows point to living oval shining cells and arrow heads point to typical apoptotic cells, condensed small sized and blue stained. (Magnification 40X)
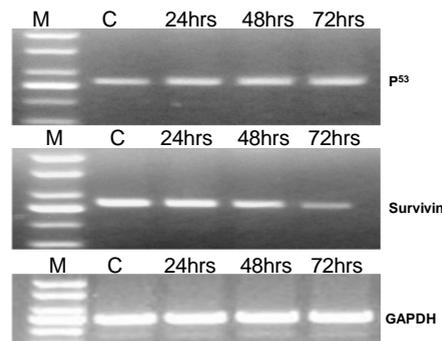
Fig. 5: Effect of aculeatiside-A on P^{53} and Survivin Expression. Cells were treated with 100 µg/ml aculeatiside-A for 24, 48 and 72 hrs then harvested and washed. The total RNA was extracted and cDNA was synthesized from total RNA (2 µg) which was amplified using the sets of primers for examining the expression of P^{53} and Survivin. -actin was used as internal control gene.

different concentrations of aculeatiside-A for different times. To clarify the mechanisms of apoptosis caused by aculeatiside-A, we detected the expressions of P[53] and surviving mRNA and protein levels of Bcl-2 and Bax after the cells were treated with 100μg/ml aculeatiside-A for 24, 48 and 72 hrs. RT-PCR and Western blot analysis revealed that both Survivin and Bcl-2 expressions were down-regulated remarkably while Bax and P[53] expression up-regulated.

Impaired apoptosis is now recognized to be a key step in tumourigenesis[11]. Recently, inducers of apoptosis have been used in cancer therapy and activation of apoptosis pathways is a key mechanism by which cytotoxic drugs kill tumor cells[12]. All these studies indicate that induction of apoptosis is now considered as an important method of assessment for the clinical effectiveness of many anti-tumor drugs[13].

Survivin, a member of apoptosis inhibitor family, is expressed in most human malignancies and implicated in mitosis regulation and preservation of cell viability[14]. Previous data have revealed that Survivin is over expressed in tumor cells which play important roles in both cell proliferation and cell death[15]. Recent studies[16&17] have shown that Survivin is over expressed and is almost always present in hepatocellular carcinoma.

Bcl-2 represents the finding member of the new and growing class of cell death inhibiting oncoproteins[18]. The first pro-apoptotic homologue of Bcl-2 family, Bax, was identified by co-immunoprecipitation with Bcl-2 protein, and the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis[19&20]. In this study, our results revealed that Bcl-2 expression was down-regulated remarkably in aculeatiside-A induced apoptosis on hepatocellular carcinoma cells.

Survivin and Bcl-2 function through different pathways in the regulation of cell apoptosis. Bcl-2 regulates apoptosis mainly by preventing cytochrome-C release from mitochondrion to cytoplasm[18&20]. Whereas, Survivin directly inhibits the activities of

Caspase-3 and 7, and block the process of apoptosis[21]. Previous studies demonstrated that the expression of Survivin was significantly associated with Bcl-2 expression and the expression of Survivin, in conjunction with Bcl-2, might cause more pronounced anti-apoptotic effects[22&23]. Therefore the expression of Survivin in cooperation with Bcl-2 is a significant prognostic parameter[24] and a new therapeutic target in cancer[23].

In the present study, we found that the expressions of both Survivin and Bcl-2 were down-regulated concurrently in aculeatiside-A induced apoptosis on the hepatocellular carcinoma cell lines. Our results agree with the findings of previous investigations, which showed that the co-expressions of Survivin and Bcl-2 play an important role in drug induced apoptosis[22&23]. The data suggest that aculeatiside-A may serve as a potential therapeutic agent for hepatocellular carcinoma. The *in-vivo* anti-tumor effects of aculeatiside-A as well as its potential clinical effectiveness need further and profound investigation (as shown in figure 6).
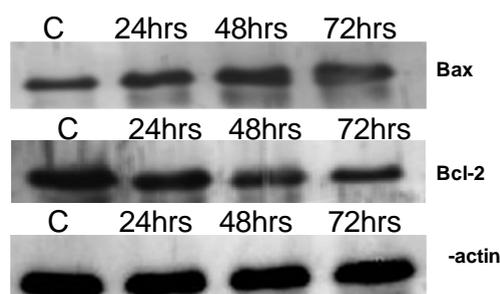
Fig. 6: Effect of aculeatiside-A on Bax and Bcl-2 Expression. Cells were treated with 100 µg/ml for 24, 48 and 72 hrs. Cells were harvested, washed and homogenized. 50 µg of protein homogenate were used from each time point. Rabbit polyclonal antibodies for Bax and Bcl-2 Were used in 1:200 dilution. Anti-rabbit secondary antibody conjugated to Alk-phospatase was used in dilution 1:3000. Data are representative of three separate experiments. -actin re-probed on the same immunoblot to sure the identity of loading.

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