

# Effects of ricin extracted from seeds of the castor bean (*ricinus communis*) on cytotoxicity and tumorigenesis of melanoma cells

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**Abstract**— Ricin has been reported as a potential therapeutic agent for the treatment of various cancers due to its potency. In this study, we succeeded in isolating and purifying total ricin from seeds of the castor bean (*ricinus communis*) from Vietnam. We also revealed that total ricin showed strong cytotoxicity against melanoma cells; IC50 at 48h was 34.1 ng/mL for SKMEL28 cells and 5.2 ng/mL for HaCaT cells. We examined the ability of total ricin to inhibit tumorigenesis of SKMEL28 cells *in vitro*. At low concentrations (< 3ng/mL) total ricin did not cause death of HaCaT or SKMEL28 cells but strongly reduced the size of SKMEL28 tumor colonies formed in soft agar. The effect of ricin on tumorigenesis was also confirmed by apoptotic and immunoblot analyses. Our results showed that treatment with total ricin (3 ng/mL) resulted in decreased ERK and p-ERK expression in SKME28 cells but did not affect expression levels of those genes in HaCaT cells. We showed that, although ricin at 1 and 3 ng/mL did not induce apoptosis of HaCaT cells, it significantly increased apoptosis of SKMEL28 up to 1.4 folds and 2.1 folds, respectively. The results from our study suggest that although ricin is listed as one of the most poisonous substances in nature, it has potential to be used as a drug for melanoma treatment.

**Keywords:** Melanoma, ricin, *ricinus communis*, tumorigenesis, cytotoxicity.

## INTRODUCTION

While malignant melanoma accounts for less than 5% of all skin cancers, it is responsible for 80% of skin cancer deaths (Gray-Schopfer et al., 2007). Outlook for patients with metastatic melanoma remains quite bleak, with a 5-year survival rate of only 5-15%. The rate has not changed significantly in recent decades despite intensive efforts to develop more effective therapies (Gray-Schopfer et al., 2007). Since melanoma is aggressive cancer with high metastatic ability, the increase in incidence is a threat to public health. It is therefore important to find new effective therapies.

Extracts derived from natural sources for cancer treatments are being increasingly studied because of their advantages, such as low price and availability. There are many natural compounds applied in treating various types of cancers, including melanoma. Previous studies have shown that natural compounds extracted from medicinal plants are capable of inhibiting cancer properties of melanoma cells by reducing the expression of ERK and/or AKT (Fagone et al., 2011; Osmond et al., 2013; Rebecca et al., 2014; Shin et al., 2008).

Ricin, extracted from seeds of the castor bean, is a glycoprotein of the lectin family (Jang and Kim, 1993; Kumar et al., 2004). Castor bean seeds contain up to

50% oil, 25% albuminoside, and other compounds, including ricin, ricinin, malic acid, and saccharide (Jang and Kim, 1993; Kumar et al., 2004). Ricin consists of two polypeptide chains (A chain: 30 kDa and B chain: 34 kDa), which link with each other via disulfide bridges (-S-S-) (Funatsu et al., 1976), and the molecular weight of ricin is about 64 kDa

Ricin is one of the most poisonous substances in nature and highly toxic in humans and animals (Funatsu et al., 1976; Lord et al., 1994). When this toxic protein enters a cell, particularly via inhalation or ingestion, it blocks the ability of cells to synthesize its own proteins, leading to cellular and organ damage. Due to its toxicity, ricin can be used for bioterrorism. Ricin can bind with high affinity to galactose residues on the cell surface and is transported through the cell membranes to cause cell death (Funatsu et al., 1976; Kumar et al., 2004; Lord et al., 1994). Although both chains contribute to toxicity, the A chain is associated with higher poisonous property and has been more studied. The A chain of ricin has been reported to be a strong immunotoxin for cells (Engert et al., 1994; Engert et al., 1990; Weidle et al., 2014).

Beside its toxic effects, ricin is also considered to have potential benefit in the treatment of a variety of cancers, including leukemia, breast cancer, cervical cancer and melanoma (Ghosh et al., 2013; LoRusso et al., 1995; Spitler, 1986; Spitler et al., 1987; Tyagi et al., 2015). Ricin A chain (but not ricin B chain and/or total ricin), as an immunotoxin conjugated with antibody, has been evaluated for melanoma treatment (Ghosh et al., 2013; LoRusso et al., 1995; Spitler, 1986; Spitler et

al., 1987; Tyagi et al., 2015). In recent years, the application of nanotechnology, such as the use of robust nanoparticle carriers for drug delivery to target cancer cells, has been rapidly developing. There are increasingly more investigations on the role of ricin as anticancer agent encapsulated into a polymer and/or liposome carrier, and used as nano drugs for the treatment of specific target cancers (Epler et al., 2012; Ghosh et al., 2013; Tyagi et al., 2015).

In this study, we focused on extracting, isolating and purifying total ricin from castor bean seeds and investigated the effects of the purified ricin on cytotoxicity and tumorigenesis of human melanoma cells *in vitro*.

## MATERIALS AND METHODS

### Castor bean seeds

Castor bean seeds were collected from Vietnam Pharmacy Institute in September 2013 and identified at the Department of Biochemistry and Plant physiology, Faculty of Biology, VNU University of Science, Vietnam National University, Hanoi, Vietnam. The castor bean seed specimen is depositing at the Biological Museum of VNU University of Science and the purified ricin is depositing at the Institute of Chemistry and Environment, High Command of Chemistry, Ministry of National Defense. We state that this purified ricin will be used for the research purposes in the laboratory only.

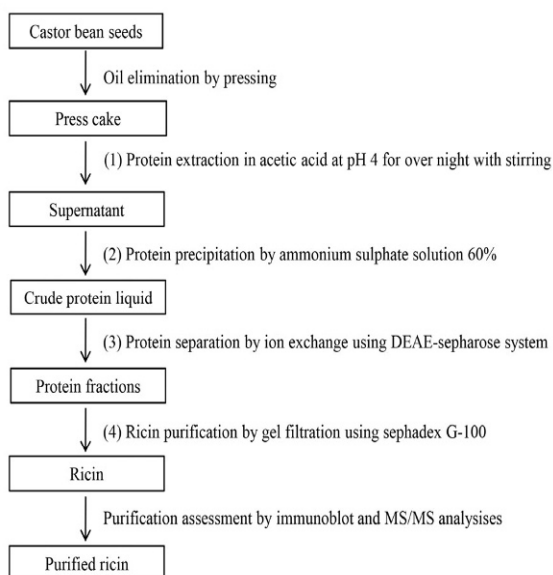
**Table 1: Isolation efficiency of total protein in ammonium sulphate  $(\text{NH}_4)_2\text{SO}_4$  solution**

Protein solutions	$\Sigma_v$ (ml)	$C_{Pr}$ (mg/mL)	$\Sigma_{Pr}$ (mg)	H (%)
Crude protein in acetic acid (5%)	300	2,25	675	-
Protein precipitation by $(\text{NH}_4)_2\text{SO}_4$ 30%	41	5,73	332,3	49,72
Protein precipitation by $(\text{NH}_4)_2\text{SO}_4$ 40%	44	6,05	347,2	51,83
Protein precipitation by $(\text{NH}_4)_2\text{SO}_4$ 50%	47	7,62	358,1	53,05
Protein precipitation by $(\text{NH}_4)_2\text{SO}_4$ 60%	50	8,59	429,5	63,62
Protein precipitation by $(\text{NH}_4)_2\text{SO}_4$ 70%	53	7,82	414,4	61,39

$\Sigma_v$ : total volume (ml)  
 $C_{Pr}$ : concentration of protein (mg/ml)  
 $\Sigma_{Pr}$ : Total concentration of protein (mg)  
H: Efficiency of protein precipitation.

### Preparation of seed extract

Castor bean seeds were cleaned and washed thoroughly with water and re-washed with distilled water. Washed seeds pressed under high pressure to remove oil. The press cake was then dried and refluxed in acetic acid 5% with 1/10 (w/v) for 12hrs to extract proteins. The extract was filtered, and the solvent was removed under reduced pressure at  $40\pm 5$  °C using a rotary flash evaporator. The scheme of the extraction procedure was presented in the figure 1 and the effectiveness of extraction procedure was presented in the table 1 (**Fig. 1; Table 1**).



**Figure 1. Procedures for isolation, purification and qualification of ricin are presented in the flow chart.**

### Protein precipitation

Ammonium sulfate with different concentrations was added into acetic acid extract solution to precipitate total protein. The examined concentrations of ammonium sulphate were 30, 40, 50, 60 and 70%. The extraction efficiencies were showed in table 1.

### Protein separation

Crude protein got in precipitation step was applied for protein separation by ion exchange using DEAE-sepharose (Amersham Biosciences). DEAE was equilibrated in 0.05M Tris-HCl buffer at pH 8.0 to create the DEAE gel. Dissolving of 20 mL crude protein solution in 50 mL DEAE gel, and stirring for 15 minutes for adsorbing of protein on the gel. Loading the protein binding gel on glass column (1.5

cmx40 cm) then washing with 100mL Tris-HCl buffer 0.05 M. Standing 30 minutes for stability before desorbing by 0.05M Tris-HCl buffer at pH 8.0 with supplementing of NaCl 0.05 M. Desorbing rate was set at 0.2 mL/minute. Protein fractions were collected with 2 mL for each. Ricin in each fraction was tested by quick test kit of Osborn, United State.

### Protein purification

Ricin was purified by gel filtration used gel sephadex G-100 (Amersham Biosciences). Firstly, gel was activated in distilled water at room temperature (1g/10mL H<sub>2</sub>O) for 6 hrs. Then, gel was loaded on glass column (2.5 cmx50 cm) and soaked phosphate buffer 0.002 M at pH 7.2 with ratio of protein solution/gel = 1:6 (v/v). Protein fractions were eluted with phosphate buffer 0.002 M at rate of 0.2 mL/minute. Protein fractions were collected with 2 mL for each fraction. Ricin in each fraction was tested by quick test kit of Osborn, United State. Standards of ricin (A chain and B chain) were purchased from Vectorlabs, USA by High Command of Chemistry, Ministry of National Defense for research purposes.

### Mass spectronic (MS/MS) analysis

Purified protein was sent to Mission Biotech Co., LTD., Taipei, Taiwan for ricin qualitative analysis.

### Cell culture

The human transformed normal skin keratinocyte (HaCaT) cells and malignant melanoma SKMEL28 cells were used. HaCaT cell was a gift from Prof. Dr. Masashi Kato, School of Medicine, Nagoya University and SKMEL28 cells were purchased from ATCC, United State. All cell lines were cultured in RPMI supplemented with penicillin (400 U/mL), streptomycin (50 mg/mL), L-glutamine (300 mg/mL) and 10% fetal bovine serum (FBS; Sigma, Deisenhofen, Germany) under a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### Cellular toxicity assay

A crystal violet assay was performed by the method described in the previous report (Yajima et al., 2012) to primarily assess the viability of cells. Briefly,  $3 \times 10^4$  cells were plated in six-well plates and cultured in the medium with or without ricin for 48hrs. The viable adherent cells were fixed with 10% formalin for 1 hour and stained with 0.1% crystal violet for 30 minutes after washing two times with PBS. The stained cells were solubilized with 0.1% SDS, and the absorbance of

the solution was measured at 595 nm by a spectrophotometer.

### ***In vitro* tumorigenesis assay**

A colony formation assay was performed to assess the development of tumor *in vitro*. Anchorage-independent growth was evaluated by the colony formation assay according to the method reported in the previous study (Thang et al., 2011). After preincubating in the medium for 48 hours,  $2.5 \times 10^4$  cells were mixed with 2 mL of 0.36% soft agar in RPMI medium, poured onto slightly solid 0.72% hard agar in RPMI medium, and then cultured for 4 weeks. Colonies exceeding 50  $\mu\text{m}$  in diameter were counted and presented as an activity of anchorage-independent growth.

### **Immunoblotting**

Immunoblotting was performed to investigate the protein expression of molecules using the method described in the previous report (Kato et al., 2010). Briefly, cells were washed twice with ice-cold PBS and lysed in 0.3 mL of lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 12.5 mM  $\beta$ -glycerophosphate, 1.5 mM  $\text{MgCl}_2$ , 2 mM EDTA, 10 mM NaF, 2 mM DTT, 1 mM  $\text{Na}_2\text{VO}_4$ , 1 mM phenylmethylsulfonyl fluoride, 20 mM aprotinin, 0.5% Triton X-100). Whole cell lysates or/and purified ricin protein were resolved on SDS-PAGE and transferred to nitrocellulose Hybond-P membranes (GE Health Sciences). After protein transfer, nitrocellulose sheets were blocked at room temperature for 60 mins with skimmed milk powder (5%) in PBS and incubated for 120 mins with different antibodies at 1:1000 dilutions.

The bound antibodies on the treated membranes were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG (Calbiochem) at 1:2000 dilutions for 90 mins at room temperature. The nitrocellulose sheets were washed three times for 5 mins with phosphate buffer saline-Tween 20 (PBST) and developed with 3,3'-diaminobenzidine. Antibodies against ricin (Agrisera – USA), ERK, p-ERK, and Tubulin (Thermo scientific) were used.

### **Annexin V apoptosis assay**

Annexin V apoptosis assay was performed to investigate the protein expression of molecules using the method described by Schutte (1998). Cells were seeded at 30 to 40% confluence in 6-cm plates. After overnight incubation, the medium was aspirated and replaced with medium with or without ricin. After 36

hours, the medium was collected. Cells were washed with PBS. Collected cells were resuspended in annexin binding buffer (Life technology) at  $1 \times 10^6$  cells/ml. Cells were stained with propidium iodide (Invitrogen) and annexin V-FITC according to the manufacturer's protocol and assayed on a FACSCanto II (BD Biosciences). The percentage of apoptotic cells was measured as the percentage of annexin V-positive cells.

### **Statistical analysis**

Statistical analysis in this study was performed according to the method described in the previous report (Yajima et al., 2012). Results from three independent experiments in each group were statistically analyzed by Student's t-test. The SPSS (version 18) software package (SPSS Japan Inc.) was used for statistical analyses, and the significance level was set at  $p < 0.05$ .

## **RESULTS**

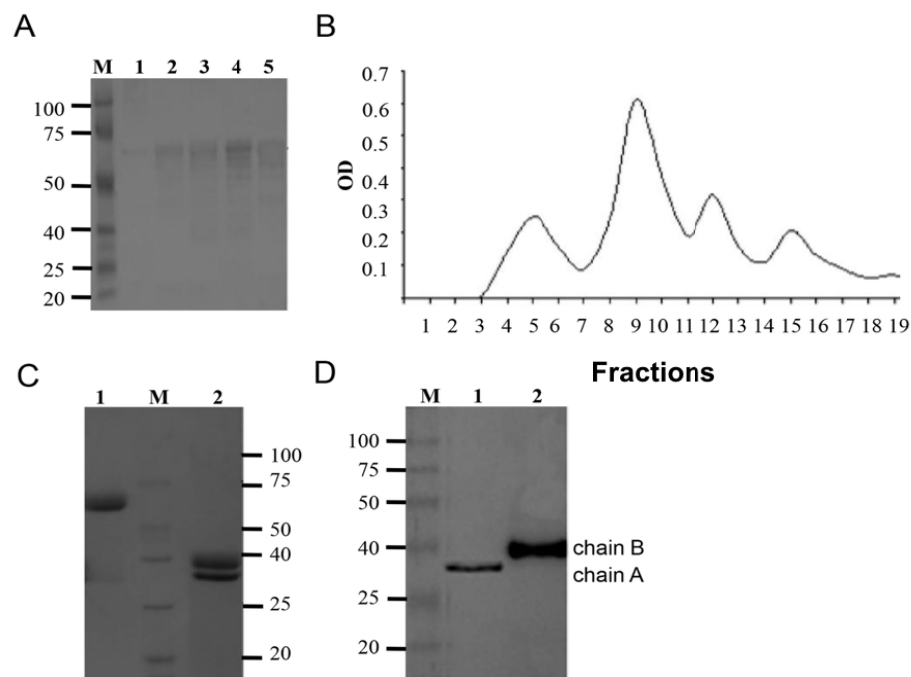
### **Isolation of ricin from castor bean seeds**

Ricin was isolated and purified using a 4-step procedure presented in Figure 1. Firstly, castor bean seeds were pressed to eliminate the oil. The press cake was dried in an oven at 40°C. Then, 100 gram of dried press cake was extracted in 1000 mL acetic acid 5% (1/10 v/v) at room temperature for 12 h with shaking at 400 revolutions/minute. After extraction, the solution was filtered through a filter paper to collect the crude protein solution (called solution I). The dried weight of crude protein extracted in acetic acid was 2.25 mg/mL (Table 1). To precipitate the total protein, ammonium sulfate was applied. After elimination of ammonium sulfate, the harvested protein was measured. Per the literature, the optimum concentration of the ammonium sulfate solution used for protein precipitation was 60% with a precipitation efficiency of up to 63%; the other ammonium sulfate solutions of 30, 40, 50 and 70% yielded protein precipitation efficiencies of 49.7, 51.8, 53.0 and 61.4%, respectively (Table 1).

Protein solution (called solution II) obtained from precipitation with ammonium sulfate were tested for the presence of ricin by quick test kit (Osborn, USA) with a limit of detection (LOD) of 50 ng. The quick test results indicated that solution II contained ricin. The

crude solution II extracts were evaluated for the presence of ricin by SDS-PAGE (**Fig. 2**); results revealed the expected bands of approximately 64 kDa (**Fig. 2A**). The band with expected size in lane 4 (protein sample from precipitation with 60% ammonium sulfate solution) had the highest intensity compared with those in the other lanes, i.e. protein

samples from precipitations with 30% (lane 1), 40% (lane 2), 50% (lane 3) and 70% (lane 5) ammonium sulfate solutions (**Fig. 2A**). These results showed that protein from solution II extracts is more likely to contain ricin protein and that 60% ammonium sulfate solution is the most suitable solution for ricin precipitation.



**Figure 2. Ricin isolation and purification.** A) Protein after precipitation with ammonium sulfate at concentration of 30% (lane 1), 40% (lane 2), 50% (lane 3), 60% (lane 4) and 70% (lane 5); B) Concentration of protein (OD) in fractions after ion-exchange using DEAE-sepharose resin; C) Ricin after purification by gel filtration using G100 sephadex; shown are total ricin without 2-ME treatment (lane 1) and with 2-ME treatment (lane 2); D) Standard ricin with A chain (lane 1) and B chain (lane 2) are presented.

### Purification of ricin from castor bean seeds

Protein solution II extracts was separated into 20 fractions (**Fig. 2B**) by ion exchange using DEAE-sepharose column chromatography. All fractions were retested for presence of ricin by quick test kit (LOD of 50ng); positive signals were detected in 4 fractions, including 8, 9, 10 and 11. Among these 4 fractions, protein concentrations (presented as OD values) of fractions 9 and 10 were highest compared to the other fractions (**Fig. 2B**). Protein from fractions 9 and 10 were mixed and referred to as solution III. This solution was used for ricin purification via gel filtration using gel sephadex G100. Purified protein solution collected after gel filtration (called solution IV) was used for immunoblot analysis with ricin

antibodies. Immunoblot analysis showed a band of 64 kDa for protein samples without 2-mercaptoethanolamine (2-ME) treatment (**Fig. 2C, lane 1**) and a double band of 30 kDa and 34 kDa for protein samples with 2-ME treatment, required to break disulfide bridges (**Fig. 2C, lane 2**). The immunoblot results for commercial standard ricin with A chain (lane 1) and B chain (lane 2) are presented in **Fig. 2D**. These results indicate that isolation and purification of total ricin protein was successful. For our 4-step procedure, isolation efficiency for each step was calculated and presented in **Table 2**, with the overall efficiency for purified ricin isolation at 9.0 %.

**Table 2: 4-step procedure for isolation and purification of ricin from castor bean seeds**

Purification steps	$\Sigma_v$ (ml)	$C_{pr}$ (mg/mL)	$\Sigma_{pr}$ (mg)	H (%)
Protein solution in acetic acid 5% (I)	1000	2,25	2250	100
Protein precipitation by $(NH_4)_2SO_4$ 60% (II)	166.5	8,59	1430.2	63,6
Protein separation ion exchange using DEAE Sepharose (III)	34	4,3	146,2	10,3
Protein purification by gel filtration using gel sephadex G100 (IV)	11	1,2	13,2	9,0

$\Sigma_v$ : total volume (ml)

$C_{pr}$ : concentration of protein (mg/ml)

$\Sigma_{pr}$ : Total concentration of protein (mg)

H: Efficiency of protein isolation and purification at each step.

### Qualitative analysis for ricin by MS/MS

Further, LC-MS/MS analysis was performed to identify ricin protein in the purified samples (protein solution IV). Gel bands from the immunoblotting experiment were excised and digested with trypsin and then analyzed by LC-MS/MS. Protein identification was evaluated using a Mascot score higher than 40; results are shown in **Figure 3**. Fragmentation of the peptide backbone in the MS/MS produced a series of b and y peaks, marked in the MS/MS. Amino acid sequence of ricin protein derived from EST gi|255587301 is presented in **Figure 3A**. Data analysis of the b and y peaks from the MS/MS spectrum led to the identification of four peptides with sequences including HEIPVLPNR (**Fig. 3B**), SFIICIQMISEAAR (**Fig. 3C**), SAPDPSVITLENSWGR (**Fig. 3D**) and LSTAIQESNQGAFASPIQLQR (**Fig. 3E**). All the sequences are parts of the ricin protein. These results confirm that the protein excised from the appropriate bands was ricin and demonstrated successful isolation and purification of total ricin from castor bean seeds.

### Cytotoxicity of ricin on human normal skin keratinocyte (HaCaT) cells and human melanoma SKMEL28 cells

Cytotoxicity of ricin was examined on HaCaT and SKMEL28 cells. The effects of ricin at various concentrations, including 0.1, 0.5, 1.0, 2.5, 5.0, 10, 100, 1000 and 10000 ng/mL, on inducing cell death of HaCaT cells after 48 h were evaluated to find the IC50 value (**Fig. 4A-H**). Results showed that the IC50 for

HaCaT cells was 5.2 ng/mL (**Fig. 4I**). Similarly, the dose-dependent effects of ricin were evaluated at various concentrations, including 0.1, 0.5, 1.0, 2.5, 5.0, 10, 100, 1000 and 10000 ng/mL. Induction of cell death of SKMEL28 cells after 48 h was also evaluated (**Fig. 5A-H**); the IC50 of ricin for SKMEL28 cells was 34.1 ng/mL after treatment with ricin for 48 h (**Fig. 5I**). These results revealed that cytotoxicity of ricin on normal skin cells was higher than that for melanoma skin cancer cells. A substance with IC50 in the range of 1-10  $\mu$ g/mL is normally considered as very toxic. Our results confirm that ricin is a highly toxic substance for cells with IC50 in the range of 5-40 ng/mL.

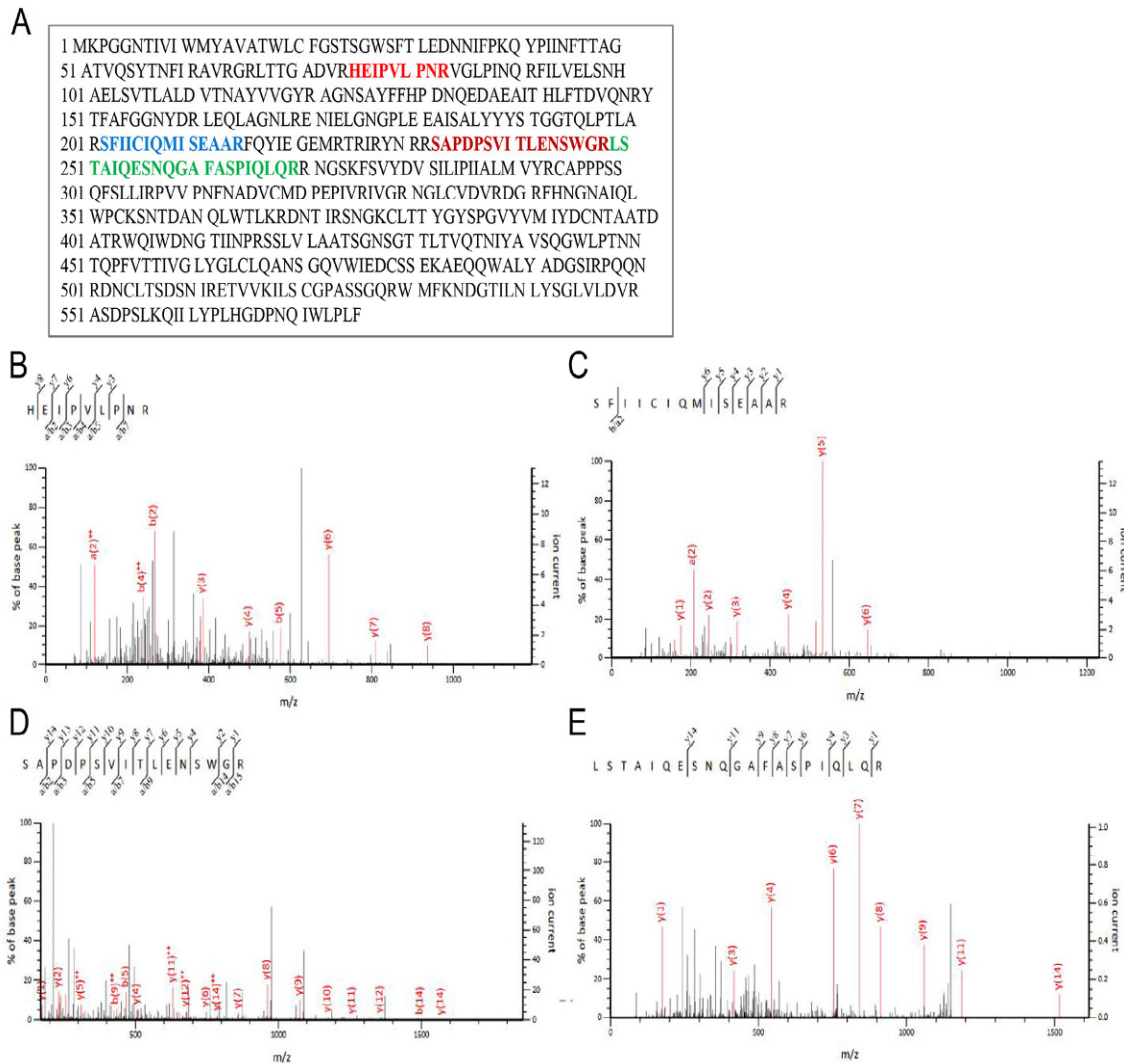
### Effect of ricin on tumorigenesis of SKMEL28 cells *in vitro*

We next investigated the role of ricin as an anticancer agent. We evaluated ricin at low concentrations (0.0, 1.0 and 3.0 ng/mL), which do not cause induce cell death of SKMEL28 cells or HaCaT cells. Our results showed that treatment with 1 or 3 ng/mL ricin did not decrease the number of colonies but did reduce the size of colonies of SKMEL28 cells formed in soft agar (**Fig. 6A, top**), compared to untreated SKMEL28 colonies (**Fig. 6A, bottom**). **Fig. 6B** shows the colonies formed in agar treated with ricin at 0, 1.0 and 3.0 ng/mL; the average sizes of colonies were also examined (**Fig. 6C**). Additionally, we also investigated the effects of ricin at concentrations of 1 and 3 ng/mL on colony formation of HaCaT cells. However, the HaCaT cells could not form colony in soft agar

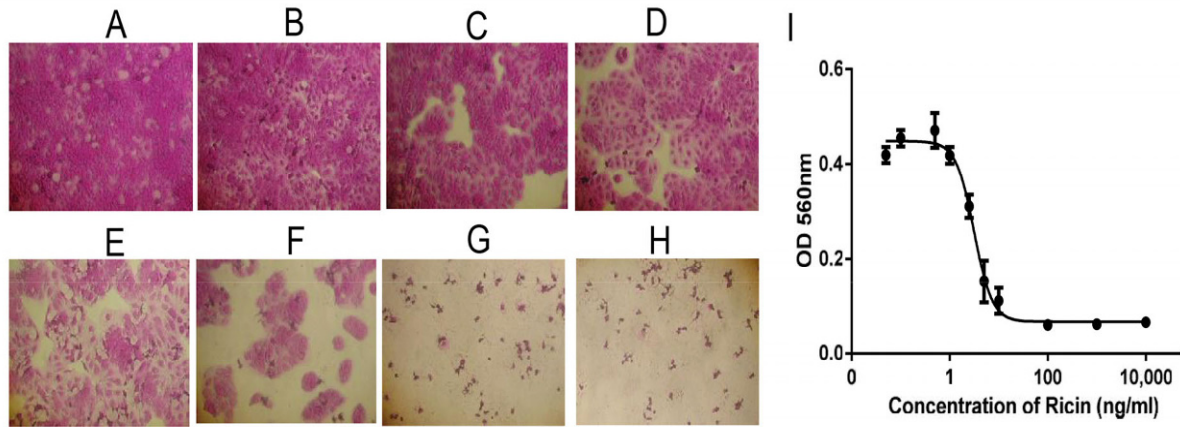


because these cells lack the typical characteristics of cancer cells (Fig. 6D). SKMEL28 and HaCaT cells were treated with or without ricin at 3.0 ng/ml for 48h before harvesting to isolate total protein. Immunoblot experiments were carried out to examine the changes of ERK and p-ERK; tubulin was used as an internal control in ricin-treated cells and compared with those in untreated cells. Our results showed that treatment SKMEL28 cells with ricin led to decreased protein expression levels of ERK and p-ERK while tubulin levels were unchanged (Fig. 6E, lane 1), when compared with those in untreated-SKMEL28 cells

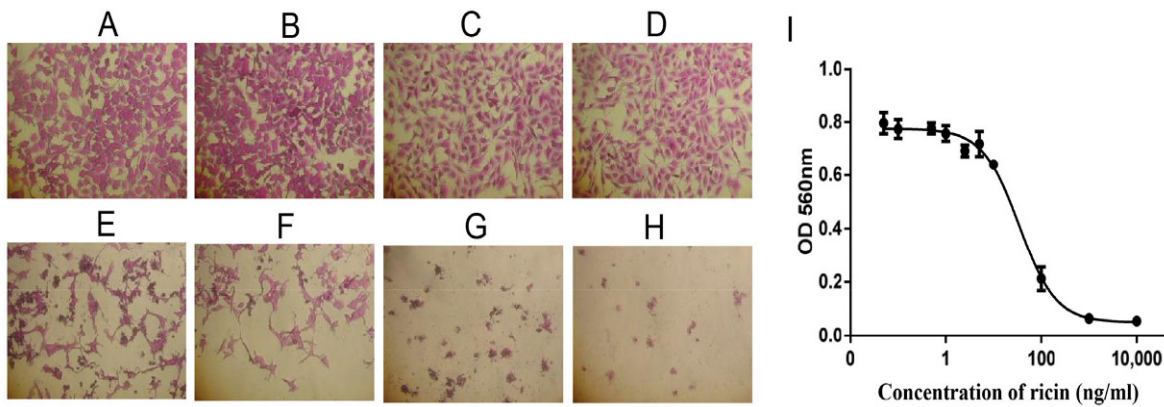
(Fig. 6E, lane 2). The expression levels of ERK and p-ERK in ricin-treated SKMEL28 cells (Fig. 6F, lane 1) were 2.38- and 3.12-fold lower than those of control SKMEL28 cells (Fig. 6F, lane 2), while treatment with ricin did not affect expression levels of ERK and p-ERK in HaCaT cells (Fig. 6G). These results suggest that ricin may reduce the tumorigenesis of melanoma skin cancer SKMEL28 cells via downregulation of ERKs (ERK and p-ERK).



**Figure 3. Peptides were fragmented by MS/MS and produced MS/MS spectrum. MS/MS spectrum for the four peptides matching with EST gi|255587301Agglutinin precursor [Ricinus communis] via Mascot Search. A) Protein sequence derived from EST gi|255587301. The matched peptides from MS/MS spectra are colored; B) MS/MS spectrum of the peptide “-HEIPVLPNRR-”; C) MS/MS spectrum of the peptide “-SFIICIQMISEAAR-”; D) MS/MS spectrum of the peptide “-SAPDPSVITLENSWGR-”; and E) MS/MS spectrum of the peptide “-LSTAIQESNQGAFASPIQLQR-”.**



**Figure 4. Cytotoxicity of ricin on HaCaT cells.** HaCaT cells were treated with ricin at concentrations of 0 ng/mL (A), 0.5 ng/mL (B), 1 ng/mL (C), 2.5 ng/mL (D), 5 ng/mL (E), 10 ng/mL (F), 100 ng/mL (G) and 1000 ng/mL (H) for 48 h. Cytotoxicity curves of ricin (at concentrations of 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10, 100, 500, 1000 and 10.000 ng/mL) on HaCaT cells after 48 h treatment (I).



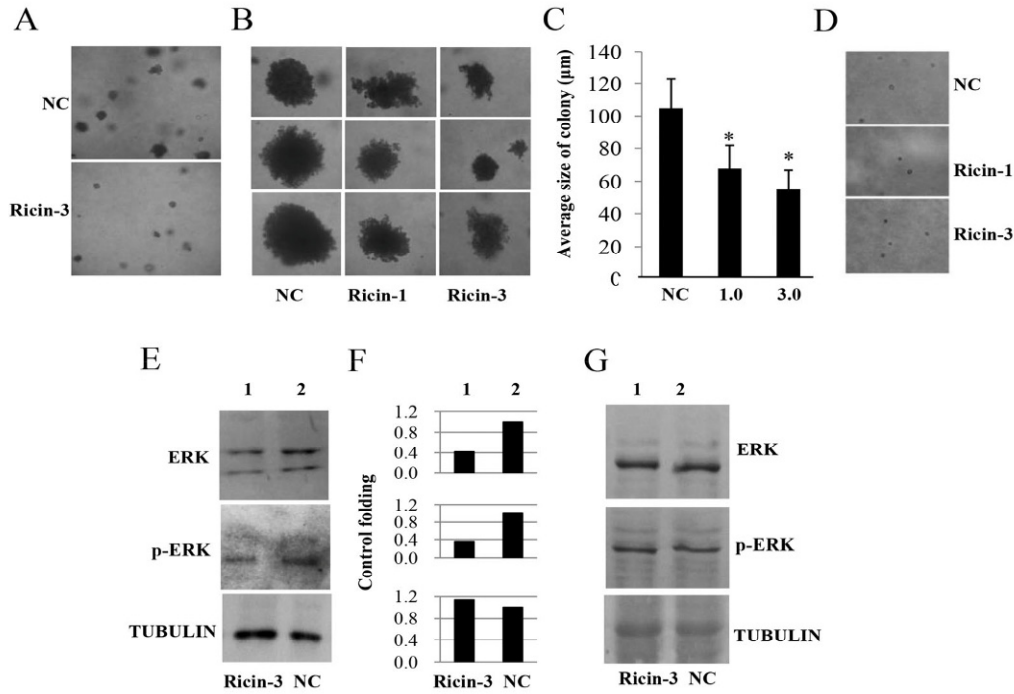
**Figure 5. Cytotoxicity of ricin on SKMEL28 cells.** SKMEL28 cells were treated with ricin at concentrations of 0.0 ng/mL (A), 5.0 ng/mL (B), 10 ng/mL (C), 25 ng/mL (D), 50 ng/mL (E), 100 ng/mL (F), 500 ng/mL (G) and 1000 ng/mL (H) for 48 h. The cytotoxicity curves of ricin (at concentrations of 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10, 100, 500, 1000 and 10000 ng/mL) on SKMEL28 cells after 48 h treatment (I).

**Effect of ricin on cellular apoptosis**

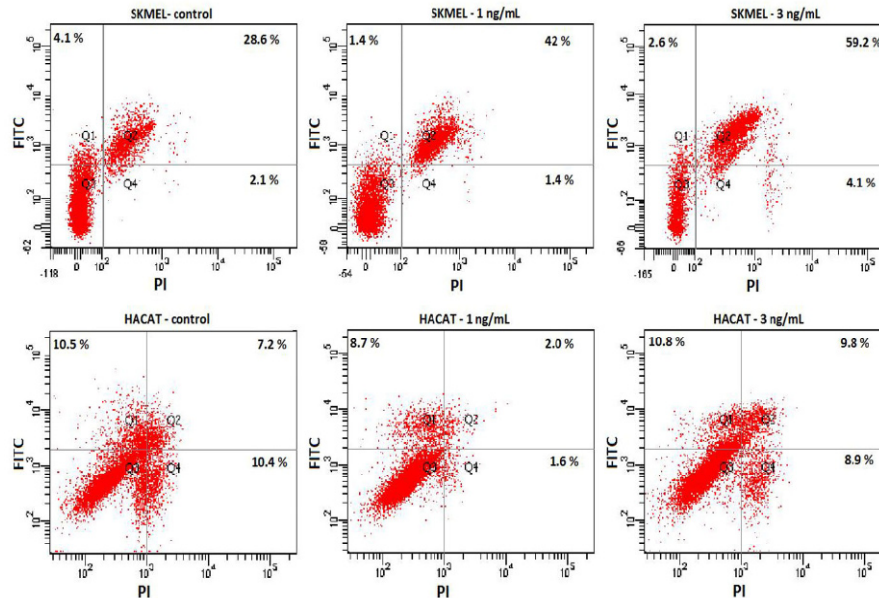
The effect of ricin on apoptosis of the HaCaT and SKMEL28 cells was investigated by using annexin V apoptosis assay on FACSCanto system. Dot blot map with intensity value of FITC (Y axis) and PI (X axis) presented the percentage of cells at different stages of early apoptosis (Q1), late apoptosis (Q2), normal (Q3) and necrosis (Q4) (Fig. 7). Total percentages of cellular apoptosis were calculated as quadrant Q1+Q2.

It was indicated that treatment with ricin at 1 and 3 ng/mL did not induce apoptosis of HaCaT cells but SKMEL28 cells. Dot blot map reflected the highest number of cells induced apoptosis in SKMEL28 cells treated with 3 ng/mL, 2.1 folds higher than the control, and 1.4 folds at 1 ng/mL. Meanwhile the percentages of either early (Q1) or late stage (Q2) of apoptosis are similar on both control and ricin-treated HaCaT cells.





**Figure 6. Effect of ricin on cell colony formation in soft agar.** A) Colonies formed in soft agar after 3 weeks in case of control SKMEL28 (up) and ricin-treated (3ng/mL) SKMEL28 (bottom) were observed under microscope at 1X magnification; Colonies formed on the soft agar after three weeks for untreated SKMEL28 cells (B) and ricin-treated cells at 1ng/mL concentration (C) and 3 ng/mL concentration (D). Cells were observed by microscopy at 10X magnification. The average size of colonies formed in soft agar with or without ricin treatment was measured and presented (E). \*, significant difference from untreated cells (control) with  $P < 0.05$  by Student's t-test. F) Expression and phosphorylation levels of ERK protein in 3 ng/mL ricin-treated SKMEL28 cells (lane 1) and untreated (control) SKMEL28 cells (lane 2), depicted in pictures (left) and graphs (right). Expression levels of  $\alpha$ -tubulin are presented as an internal control. G) Colonies of HaCaT, with or without ricin treatment, formed in soft agar are shown. H) Expression and phosphorylation levels of ERK protein in 3 ng/mL ricin-treated HaCaT cells (lane 1) and untreated (control) HaCaT cells (lane 2) are depicted. Expression levels of  $\alpha$ -tubulin are presented as an internal control.



**Figure 7. Effect of ricin on apoptosis of the cells.** Apoptotic analyses for HaCaT and SKMEL28 cells treated with ricin at 1 ng/mL and 3 ng/mL are presented.

## DISCUSSION

Ricin is comprised of a catalytic A subunit (A chain) and a lectin B subunit (B chain) which mediates cellular uptake. For ricin, these chains are referred to as ricin toxin A-chain and ricin toxin B-chain, respectively (Ready et al., 1986). Toxicity of ricin is expressed as its ability to chemically inactivate the eukaryotic ribosome by hydrolysis of a single adenine base (A4324) on the sarcin-ricin loop (SRL) of the 28S rRNA of the large subunit. This leads to loss of ribosomal activity and finally results in cell and/or tissue death (Endo and Tsurugi, 1987; Endo et al., 1988). Ricin A chain has been long reported to be a potential drug for various cancers, including leukemia, Hodgkin's diseases, breast cancer, cervical cancer and melanoma (Ghosh et al., 2013; LoRusso et al., 1995; Spitler, 1986; Spitler et al., 1987; Tyagi et al., 2015). However, other previous reports have shown that ricin B chain and total ricin also have antitumor activities in cervical cancer (Sadraeian et al., 2013; Liao et al., 2012).

Although ricin has potential as an anticancer agent, its specificity is one of the greatest challenges for the application of ricin in cancer therapy. Therefore, for practicality, ricin is mainly used in immunotoxic therapy as monoclonal antibody-conjugated forms to increase the specificity of the drug towards target cells and to decrease side effects (Epler et al., 2012; Alipour et al., 2013; Buonocore et al., 2011). Recently, developments in nanotechnology, especially nanoliposomes and its applications, have led to investigations of drug delivery systems to target cancer cells. From this, many new generations of liposomal ricin have been developed. Several reports have shown that ricin encapsulated into nanoliposomes showed antitumor abilities in various types of cancers (Epler et al., 2012; Tyagi et al., 2015) Buonocore et al., 2011). This led to our interest in the design and development of a nanoliposome system carrying ricin for future targeted treatment of cancers, including melanoma.

Melanoma is one of the most aggressive cancers in the world, particularly in Europe, United State and Australia (Gray-Schopfer et al., 2007; Meier, 2005). So far, there is no effective drug for treating melanoma. In the past, ricin A chain, but not ricin B chain and/or total ricin, was used as an immunotoxin for treatment of melanoma (Spitler, 1986; Spitler et al., 1987).

However, almost all these studies were mainly focused on investigating the effects of ricin on metastatic melanoma in animal models rather than on cell lines. There have been only a few studies using melanoma cell lines for research; these studies showed that ricin-A-chain immunotoxin could penetrate into tumor spheroids and kill cells, though the mechanism was unclear (Kikuchi et al., 1992).

In this study, for the first time, the toxic effects of total ricin on melanoma SKMEL28 cells and normal skin keratinocyte HaCaT cells will be evaluated and the IC50 values determined for purified ricin on these cells. We showed that purified total ricin could decrease the size of colonies of SKMEL28 cells. In general, during development and division stages, a colony should be in rounded shape with a bright color in the outer area. This reflects that cells in the outer area are still alive (bright color as indication) and dividing equally in every direction (round shape as indication). However, under the effect of a drug, cells may undergo apoptosis and colonies will be broken down, leading to change in shape and color. A darkening of color in the outer area means that cells in the outer area are dying from apoptosis or necrosis. Results in Figure 6B-D showed that treatment with ricin at low concentrations not only reduced the size of the colonies but caused a subtle change in shape of colonies and a slightly darker color in the outer area of those colonies, compared to control colonies (**Fig 6B**).

Furthermore, our data reveal that total ricin may reduce the tumorigenesis of cells *in vitro* via downregulation of ERK and p-ERK protein levels. It is well-known that the development of cancer, including melanoma, is related to phosphorylation/activation of two signaling pathways: MAPK (RAS/RAF/MEK/ERK) (Meier, 2005). Both ERK and phosphorylated ERK (p-ERK) are key molecules which contribute to regulation of proliferation, differentiation and invasion of cancer cells (Gray-Schopfer et al., 2007; Meier, 2005). It was reported that increased expression of ERK appears in more than 90% of melanoma cases (Cohen et al., 2002) and inhibition of expression of molecules such as RAF (BRAF) and MEK leads to reduced expression of ERK (McCubrey et al., 2007). Previous reports have also shown that inhibition of ERK/p-ERK will result in the reduction of colony formation ability of cancer cells. ERK is a downstream molecule of the MAPK signaling, which is known to regulate the

proliferation of cells and colony formation *in vitro* and tumor formation *in vivo* (Gray-Schopfer et al., 2007; Meier, 2005). In accordance with these previous findings, our results showed the relationship between downregulation of ERK/p-ERK and reduction of colony size in soft agar (Fig. 6). Another interesting thing is the fact that ricin at low concentrations (1 and 3 ng/mL) barely affected apoptosis of HaCaT but significantly induced apoptosis of SKMEL28 cells (Fig. 7). These results suggested that total ricin might be a good candidate for melanoma treatment.

## CONCLUSIONS

Although ricin can be used as a drug for cancer treatment, it is impossible to purchase from commercial sources because of its poisonous property and potential application for bioterrorism. Therefore, ricin must be isolated and purified directly from castor bean seeds in order to study its biological effects in Vietnam. In this study, ricin was extracted by acetic acid and precipitated by 60% ammonium sulfate. Ricin was then purified by ion exchange chromatography using DEAE-sepharose system and gel filtration using sephadex G100. Purified ricin was confirmed by Western Blot analysis and MS/MS analysis. In this study, we have shown the successful isolation and purification of integral ricin (both RT-A and RT-B chains) from castor bean seeds, with an efficiency of up to 9.0%. We investigated the effects of the purified ricin at low concentrations (< 3ng/mL) on cytotoxicity of normal skin HaCaT cells and melanoma skin cancer SKMEL28 cells. Our results showed that ricin at very low concentrations (1ng/mL and 3 ng/mL) have the potential to decrease the size of tumors formed in soft agar and increase apoptosis of SKMEL28 cells but not HaCaT cells via downregulation of ERK and p-ERK, which play important roles in cell survival, differentiation, tumorigenesis and invasion. Our results suggest that ricin, in its integral form, can be used as a drug for melanoma treatment. However, further studies are needed to optimize the dose and form of usage (e.g. as a single drug or drug-coated with liposome/polymer) to increase specificity and reduce side effects.

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## Competing interests

The authors declare that they have no competing interests.

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