Chitosan extracted from the Persian Gulf chiton shells: Induction of apoptosis in liver cancer cell line

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Abstract

Here for the first time, we investigated the cytotoxic effects of the chitosan extracted from the Persian Gulf Chiton shell (*Acanthopleura vaillantii*) on liver cancer cell line (HepG2). Chitosan extraction was implemented following this method: chitin was produced by demineralization and deproteinization procedure, and the extracted chitin was converted into soluble chitosan using deacetylation method. The cytotoxic effects of extracted chitosan were evaluated using four different tests, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Annexin V-FITC, propidium iodide (PI) staining, 4',6-diamidino-2-phenylindole (DAPI) staining, and Caspase activity analysis. The IC_{50} inhibitory concentrations of chitosan were obtained at 250 µg/mL after 24 h. Chitosan clearly inhibited the growth of hepatocarcinoma cells *in vitro* in a dose-dependent manner. For detecting the induced cell apoptosis, $HepG_2$ cells were treated with 125, 250 and 500 µg/ml of chitosan for 24 h. According to the result of Annex in V/PI kit, in 125, 250, and 500 µg/ml of chitosan, 28.2, 49.1, and 83.3% of $HepG_2$ cells undergone late apoptosis, respectively. The morphology of treated cells by DAPI staining showed non uniform plasma membrane and DNA fragmentation compared to untreated cells with perfect nucleus. The analysis of cell cycle using flow cytometry demonstrated that the rate of sub-G1 peak was increased to 52.7%. Both caspase-3 and -9 activities increased by the extracted chitosan, but it was only significant for caspase-3. The results of the present study suggested that the extracted chitosan has efficient cytotoxicity on $HepG_2$ cells. Therefore, the extracted chitosan from the shell of the Chiton may be considered as a futuristic natural product regarding the treatment of liver cancer.

Keywords: Chiton, Chitosan, Persian Gulf, Natural product, Apoptosis

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Introduction

In recent years, oceans and seas have been considered as a source of compounds with special structure and biological activities (Dhinakaran *et al*., 2012). Marine invertebrates (e.g. Mollusca) are one of the main groups of marine organisms. Nowadays, various ranges of secondary metabolites have been extracted from marine invertebrates which are extremely useful in marine medicines' production (Zoysa, 2012). Often, polyplacophora is known as Chiton and a live fossil, because they are an ancient group of Mollusca (Ávila and Sigwart, 2013). They maintain multi-layer shells since million years ago (Sigwart, 2009) and have more than 940 live species. Moreover, about 430 fossil specimens have been identified worldwide (Stebbins and Eernisse 2009; Weaver *et al*., 2010). The shells include eight aragonite segments, which are firmly attached to the rocks by the foot and their radula is adapted to their way of feeding the soft part of their body that is being used in the food industry (Connors *et al*., 2012).

Natural products are secondary bioactive metabolites with clear biological effects which make them appropriate candidates for discovering anti-tumor medicine (Hussain *et al*., 2012). Investigation on new medicine seems important in cancer treatment, because most cancers are resistant to chemotherapeutic medicine (Arizza, 2013). In addition, the high toxicity which is usually observed in some cancer chemotherapeutic medicines can bring about critical side effects. Therefore, the demands for new anti-cancer medicines derived from natural compounds with the lowest toxicity, slightest side-effects, and higher therapeutic efficiency are increasing (Demain and Vaishnav, 2011).

The liver as a vital organ plays great roles in several contexts, including the whole body metabolism activities, important bio-chemical conversion, energy homeostasis, etc., (Mueller *et al*., 2012). There are various liver diseases and they include alcoholic liver, fibrosis, cirrhosis, and liver cancer (Xu *et al*., 2010; Ibrahim *et al*., 2011). Today, medical advances in the treatment of the liver cancer are not enough for prevention or reduction of tumor spread. Therefore, the overall survival rate of patients with various liver cancer has not been reported to be significantly improved in the last two decades (Gao *et al*., 2012). Surgery and chemotherapy have been known as the conventional therapeutic procedures that are used despite their high side effects and low response rates (Park and Kim, 2010). The role of substances derived from natural source in reducing malignant effects has been improved by recent studies. New evidence shows that the evaluation of the role of natural substances *in vivo* and *in vitro* has been increased compared to previous years (Skropeta, 2014).

Chitin is a naturally plentiful polymer and the second most common polysaccharide on earth after cellulose. This polymer is hard and consists of a linear chain of linked 2-acetoamido-2-deoxy-β-D-glucopyranose units (Islam *et al*., 2011; Ocloo *et al*., 2011) Chitin is

usually found in the shell of crustaceans, the cuticles of insects, and the cell walls of fungi (Sagheer et al., 2009).

Chitosan is derived from deacetylation of chitin; it is a linear polysaccharide that consists of (1-4)-linked 2-amino-2-deoxy-b-D-glucopyranose. One of the most common applications of chitosan is in biomedical and pharmaceutical products (Bobu *et al*. 2011). Chitosan and their derivatives have been examined in terms of their utility as medicine carriers for anti-cancer treatment. Biological properties of chitosan, including biocompatibility and biodegradability depend on the deacetylation degree. Chitosan with a deacetylation degree of 50 to 60% is a suitable issue for lysozyme-catalyzed degradation (Kato *et al*., 2005). Therefore, it seems that increasing deacetylation degree of chitosan up to 90% is useful for anti-cancer applications. The unique specifications of the chitosan nanoparticles could bring a higher dependency on negatively charged biological membranes and site-specific targeting *in vivo*. Chitosan nanoparticles have dose-dependent inhibitory activity on the proliferation of various tumor cell lines with low toxicity against normal human liver cells (Aruna *et al*., 2013). According to various studies, chitin and chitosan have been widely applied in medicine and pharmaceuticals. They increase the persistent motion for the expansion of safe and effective drug delivery systems, because of their unique physicochemical and biological characteristics (Li *et al*., 2013). One of the new applications of chitosan is the direct use of extracted chitosan from various sources on cancer cell line, such as extracted chitosan from some of the crustacean, insect, and fungi. Therefore, in this paper, the cytotoxic effect of chitosan extracted from the Persian Gulf Chiton shells on liver cancer cell line (HepG2) was reported for the first time. This study may hopefully provide novel natural resources for chitosan.

Materials and methods

Extraction of chitin and preparation of chitosan

Chitons (*A. vaillantii*) were collected from the rocky coast of southern Qeshm Island of the Persian Gulf and their shells (aragonite) were then separated from the soft tissues and shipped to the laboratory in the wet ice immediately.. Extraction process of chitin was divided into 3 steps (Fig. 1):

A: Demineralization was carried out at room temperature using 1 M hydrochloric acid solution (40 mL/g) for 3 h.

B: Deproteinization of chitin was carried out using 1 M sodium hydroxide solution (20 mL/g) at 70°C to obtain chitin.

C: Deacetylation of chitin was carried out by refluxing chitin in 45% sodium hydroxide solution (15 mL/g) at 110ºC for 24 h to obtain chitosan.

To solve the chitosan, 2 mL of the obtained chitosan was dissolved in 250 µL of acetic acid glacial 2%. Then, the different doses of chitosan were made for cytotoxic tests using serial dilution method.

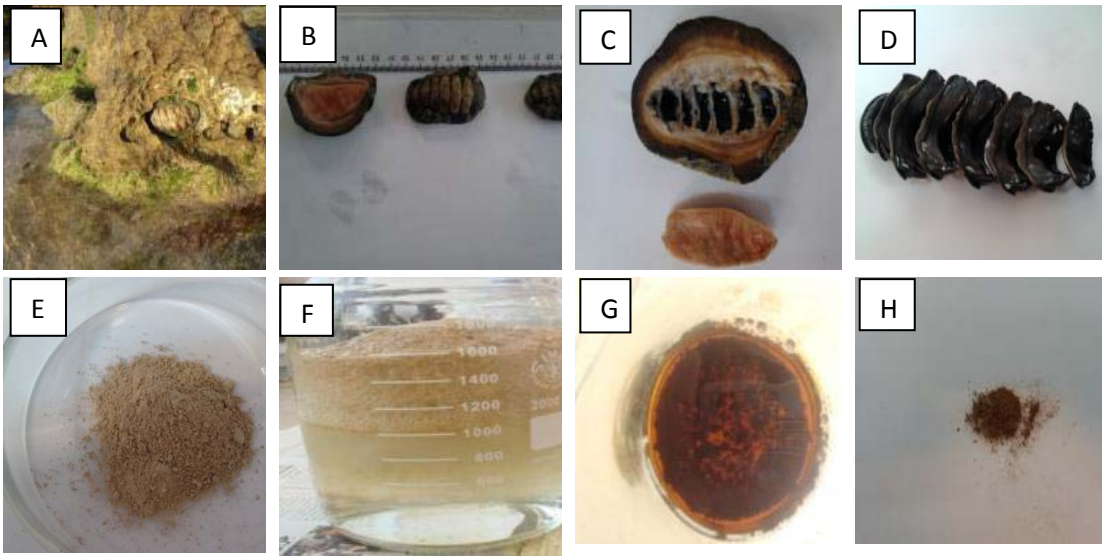


Figure 1: Different stages of chitosan preparation. A: Chiton attached to rock in the Persian Gulf. B: Identification of Chiton in lab. C: Removal of radula. D: Separation of shells (aragonite). E: Powder of aragonite after grinding. F: Demineralization stage. G: Deproteinization stage. H: Extracted chitosan.

Materials for cell culture

$HepG_2$ cancer cells were purchased from national cell bank of Iran (NCBI). Dulbecco's modified eagle's medium (DMEM) were purchased from Bio Idea (Iran), Trypsin/EDTA (1X) and fetal bovine serum (FBS) were provided by GIBCO (USA), penicillin/streptomycin, and phosphate buffered saline (PBS) were purchased from PAA (Austria). AnnexinV-FITC and caspases 3 and 9 kits were bought from Abcam (UK), and 3-(4, 5-dimethylthiozol-2-il) 2,5dipheniltetrazoliumbromide (MTT) was purchased from SIGMA (USA).

Cell culture

The $HepG_2$ cell line was obtained from Pasteur Institute of Iran. Cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin, and then incubated at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

At first, the $HepG_2$ cells were seeded in 96-well plates and treated with different concentrations of chitosan solution for 24 and 48 h. Then, 5 mg of MTT powder mixed with 1 ml PBS was added to the wells and incubated at 37°C in the dark for 4 h and dimethyl sulfoxide (DMSO) was added to totally dissolve the formazan crystals. The absorbance of each well was measured at a wave length of 560 nm with a Spectrophotometer (Shen *et al*., 2009). The cell viability inhibition was calculated using the following equation:

Cell viability (%) =Absorbance in test wells / Absorbance in control wells × 100

Annexin V-FITC

The appearance of Phosphatidylserine (PS) on the extracellular side of the membrane was evaluated using Annexin V/PI method. For detection of induced cell apoptosis, $HepG_2$ cells were treated with 125, 250 and 500 µg/mL of chitosan for 24

h. Then, the cells were suspended in 500 µL 1X binding buffer after being treated with different concentrations of chitosan and centrifuged according to the company's protocol. Then, 5 µL of Annexin V-FITC and 5 µL of Propidium iodide (PI) were added to centrifuged cells and then they were placed at room temperature in darkness for five minutes. Finally, they were analyzed on flow cytometer (Cheng *et al*., 2012; Guan *et al*., 2012).

Analysis of cell cycle by flow cytometry

To evaluate the effect of the extracted chitosan on cell cycle, the $HepG_2$ cells were first seeded in 6-well plates and then 2.5 ml of the respective culture medium were added. Next day, the medium was replaced by chitosan with concentrations of 125, 250 and 500 µg/mL, in 2.5 mL of fresh culture medium. An additional solution without chitosan was carried out as a control group. After 24 h, cells were trypsinised and separated from the plate and ready for flow cytometry. In general, after the cell was collected and washed with PBS, 700 µL PI solution was added to the wells and incubated at 37ºC for 20 min in the dark. Then, different concentrations of the solution were sorted in various micro-tubes and analyzed using flow cytometry *(Liu et al., 2010; Cheng et al*., 2012).

4',6-Diamidino-2-phenylindole (DAPI) staining

Apoptotic morphology was monitored in DAPI stained cells. The $HepG_2$ cells were grown for 24 h on cover slips in 35-mm dishes in the presence of 125, 250, and 500 µg/mL chitosan and absence of chitosan as a control group. Cover slips were washed twice using PBS fixed with 1000 mL of methanol for 5 min and incubated with 10 µg/mL DAPI for 10 min in the dark. Cells were washed with PBS and observed under a fluorescent microscope (Lu *et al*., 2011).

Caspase activity analysis

The caspase-3 and caspase-9 were significant in the hanging phase of the apoptosis. Cells were lysed and then tested for protease activity by adding a caspase specific peptide that wasconjugated with a chromophore p-nitroaniline (pNA). The assay was performed using a caspase-3 or caspase-9, apoptosis detection, Colorimetric Bio Assay Kit (Abcam-UK) based on the protocol of the company. $HepG_2$ cells (3×10^5) were treated with three concentrations of chitosan, including 125, 250, and 500 µg/mL for 24 h. The control and treated cells were resuspended in 50 µL of the cell lysis buffer (supplied with the kit) and incubated on ice for 10 min. Supernatants (cytosolic extract) were centrifuged and moved to new tubes and kept on ice. The caspase-9 assay was done according to the supplied kit protocol. 50 µL of 2X reaction buffer (containing 10 mM dithiothreitol [DTT]) was added to each sample. Five µL of LEHD-pNA substrate (4 mM; 200 µM final concentration) was added and incubation was done at 37°C for 2 h and thereafter absorbance was read at 405 nm (Wimardhani *et al*., 2014).

Statistical analysis

Data analyses were performed using Statistical Package for Social Sciences (SPSS) 16 software and one-way analysis of variance (ANOVA) to determine the significance among the groups. All tests were assumed to be statistically significant at $p<0.05$.

Results

Monitoring cell morphology

First observation proved cytotoxic effects of the extracted chitosan was changing in $HepG_2$ cells morphology as the cells were shrunk after being exposed to the chitosan solution which showed reduced cell volume, plasma membrane distortion, nuclear condensation, and apoptotic bodies. All these morphological changes are known as the apoptotic characteristics (Fig. 2).

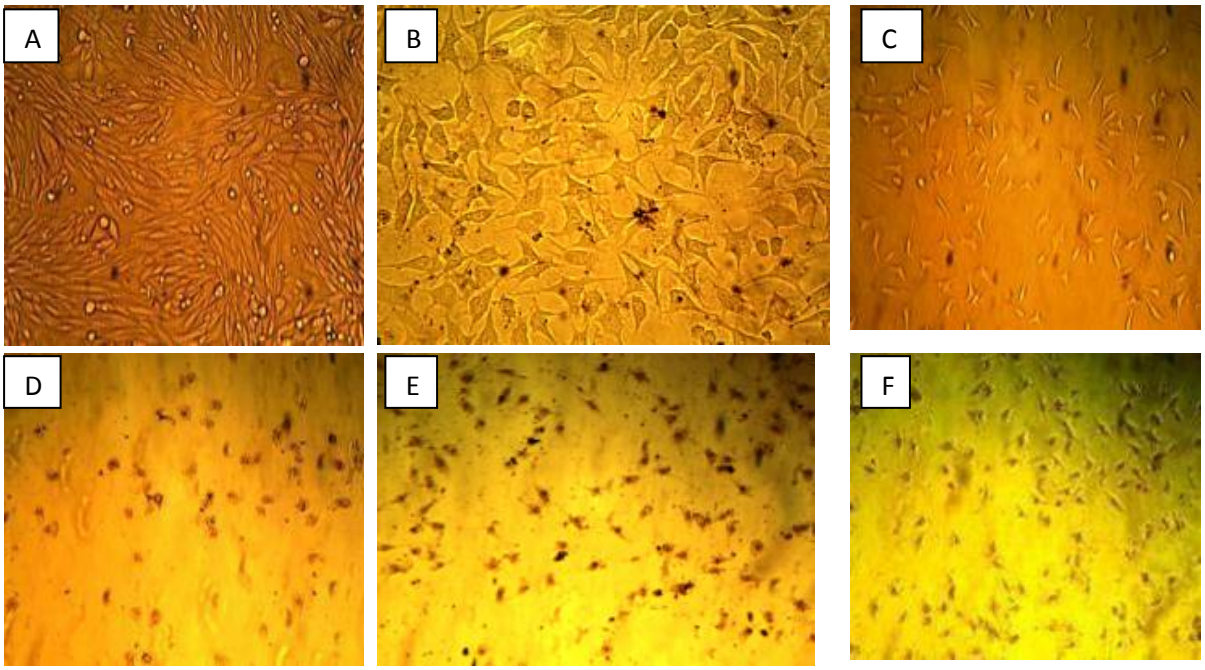
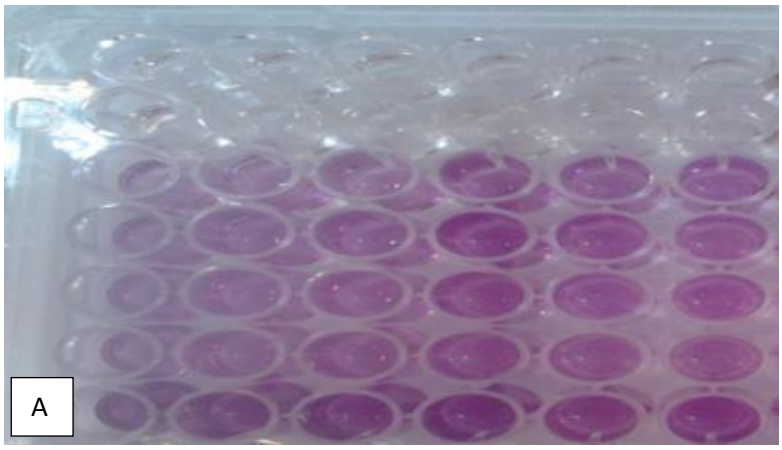


Figure 2: Effects of chitosan on cytomorphological changes of $HepG_2$ cells. Cancer cell line $HepG_2$ was treated with different concentrations of chitosan. A& B: control (without chitosan), C: sham (serial diluted method, only acetic acid glacial 2% named "sham" was used alongside the control group to study the only effect of extracted chitosan on HepG2), D: 125 µg/mL of chitosan, E: 250 µg/mL of chitosan, F: 500 µg/mL of chitosan.

MTT assay

The second test was MTT assay used to evaluate cytotoxic effects of the extracted chitosan. $HepG_2$ cells were categorized into three groups: the control, sham, and treated groups. The half maximal inhibitory concentration (IC_{50}) of chitosan on $HepG_2$ was calculated using linear regression. The IC_{50} inhibitory concentrations of chitosan obtained 250 µg/mL after 24 h ($p<0.05$) (Fig. 3). Chitosan clearly inhibited the growth of hepatocarcinoma cells *in vitro* in a dose-dependent manner and showed statistically significant differences in inhibition rates.



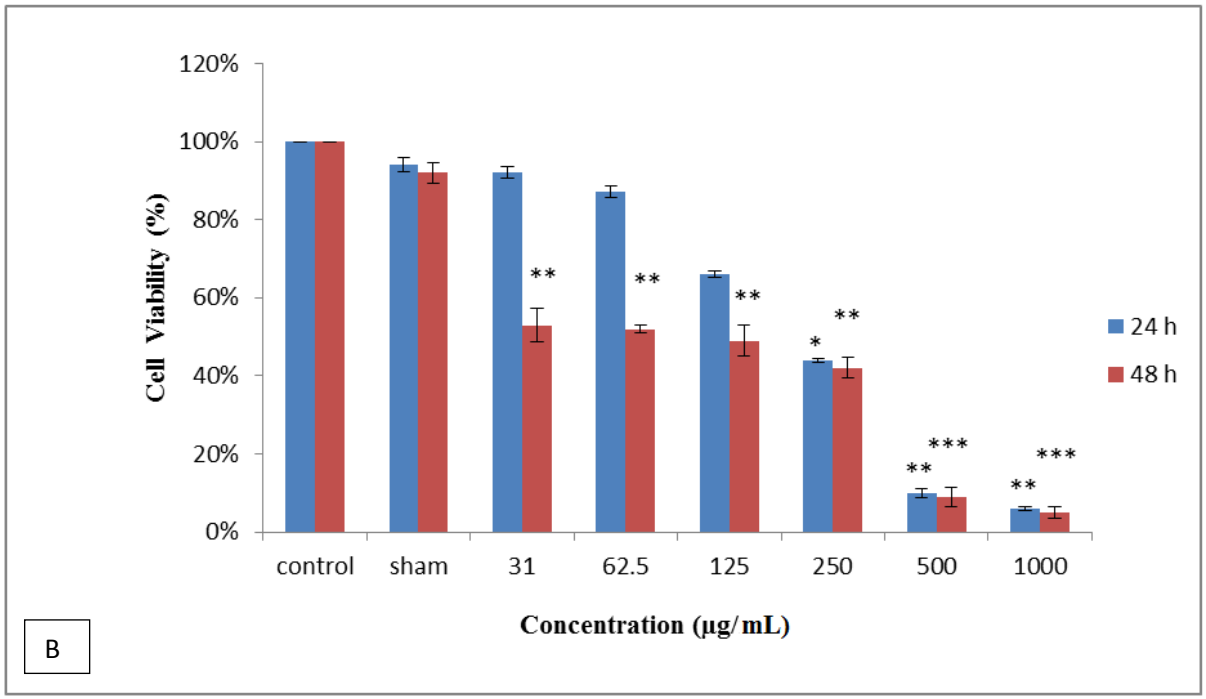


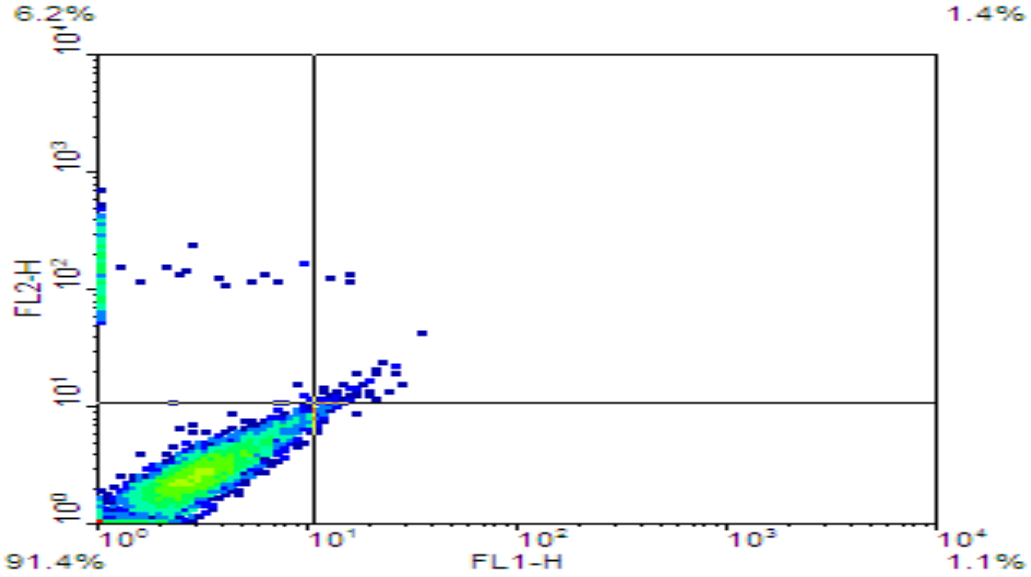
Figure 3: A: The photo is from MTT assay in 96-well plates. B: Percentages of cell viability $HepG_2$ cell line treated with different concentrations of chitosan after 24 and 48 hours using MTT assay. The data were represented as mean ± SD. *p<0.05, **p<0.01 and ***p<0.001 were considered significant.

Table 1: Percentages of cell viability $HepG_2$ cell line treated with different concentrations of chitosan after 24 and 48 hours using MTT assay. The data were represented as mean ± SD.

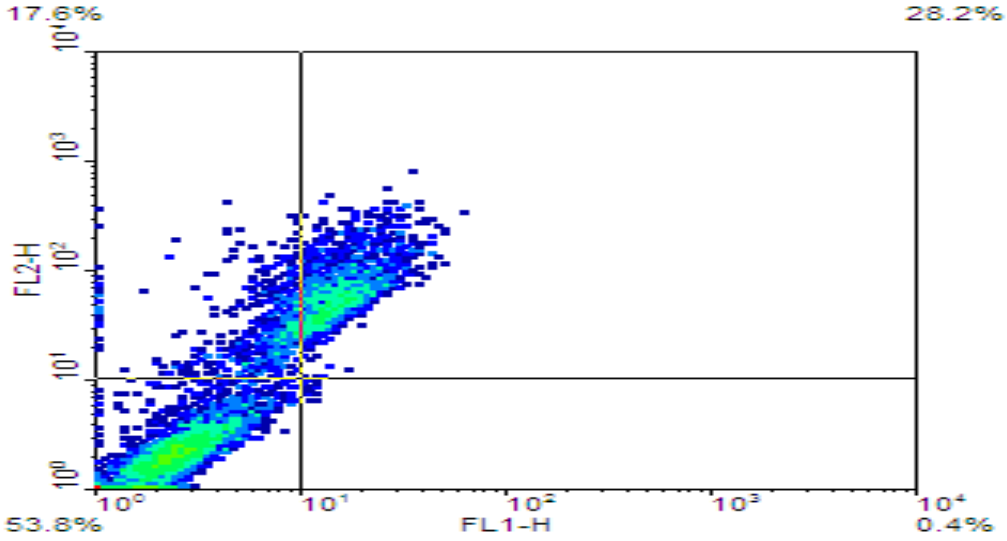
Concentration(µg/mL)	Cell viability (%) after 24 h	Cell viability (%) after 48 h
Control	100±0	100±0
Sham	94±0.017	92±0.025
31 µg/mL	92±0.015	53±0.041
62.5 µg/mL	87±0.014	52±0.011
125 µg/mL	66±0.008	49±0.039
250 µg/mL	44±0.004	42±0.026
500 µg/mL	10±0.010	9±0.024
1000 µg/mL	6±0.006	5±0.015

Flow cytometry analysis for apoptosis detection

The third test for cytotoxic effects was flow cytometry analysis by Annexin V-FITC. For detection of induced cell apoptosis, $HepG_2$ cells were treated with 125, 250, and 500 µg/mL of chitosan for 24 h. Then, the cells were stained with Annexin V-FITC according to the manufacturer's manual and then analyzed using flow cytometry. The apoptosis of $HepG_2$ cells induced by chitosan was verified through flow cytometry. Early apoptosis and late apoptosis/necrosis cells were distinguished through Annexin V-FITC staining. According to the results of this study, cells underwent late apoptosis in 125, 250 and 500 µg/mL of chitosan, 28.2, 49.1 and 83.3% of $HepG_2$, respectively (Fig. 4).



(A)



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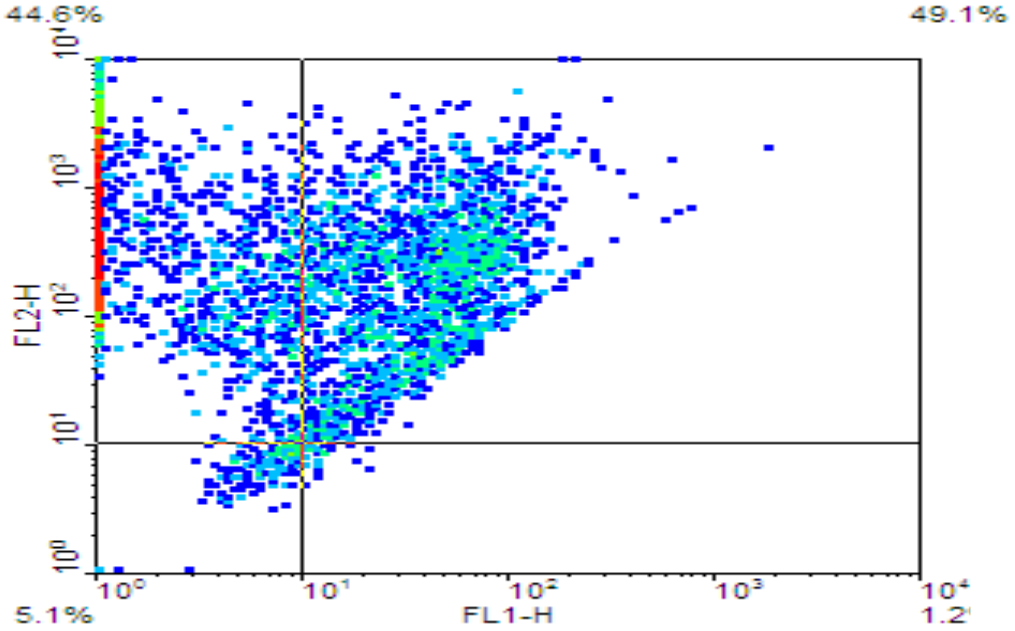

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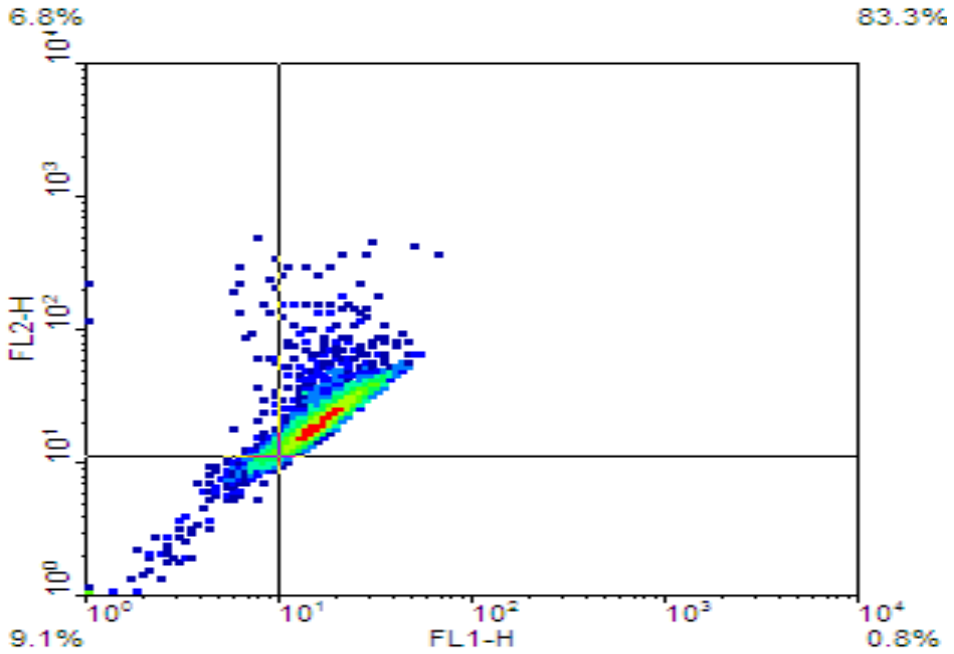


(D)

Figure 4: Apoptosis induced by chitosan on $HepG_2$ cells. (A) Control (B) 125µg/mL (C) 250 µg/mL (D) 500µg/mL of chitosan conducted by Annexin V/PI assay.

DAPI staining

The fourth test used in this study to evaluate cytotoxic effects of the extracted chitosan was DAPI staining. The morphological changes of apoptotic cells were observed under a fluorescence microscope after DAPI staining. Fig. 5 shows significant morphological changes in nuclear chromatin after chitosan treatment for 24 h. $HepG_2$ cells treated with different concentrations of extracted chitosan showed non uniform plasma

membrane and DNA fragmentation compared to the untreated cells with perfect nucleus. Typical apoptotic nucleus changes (chromatin condensation, nuclear fragmentation) were observed after chitosan treatment. It is noteworthy that fluorescent staining is a common procedure for detection of apoptosis.

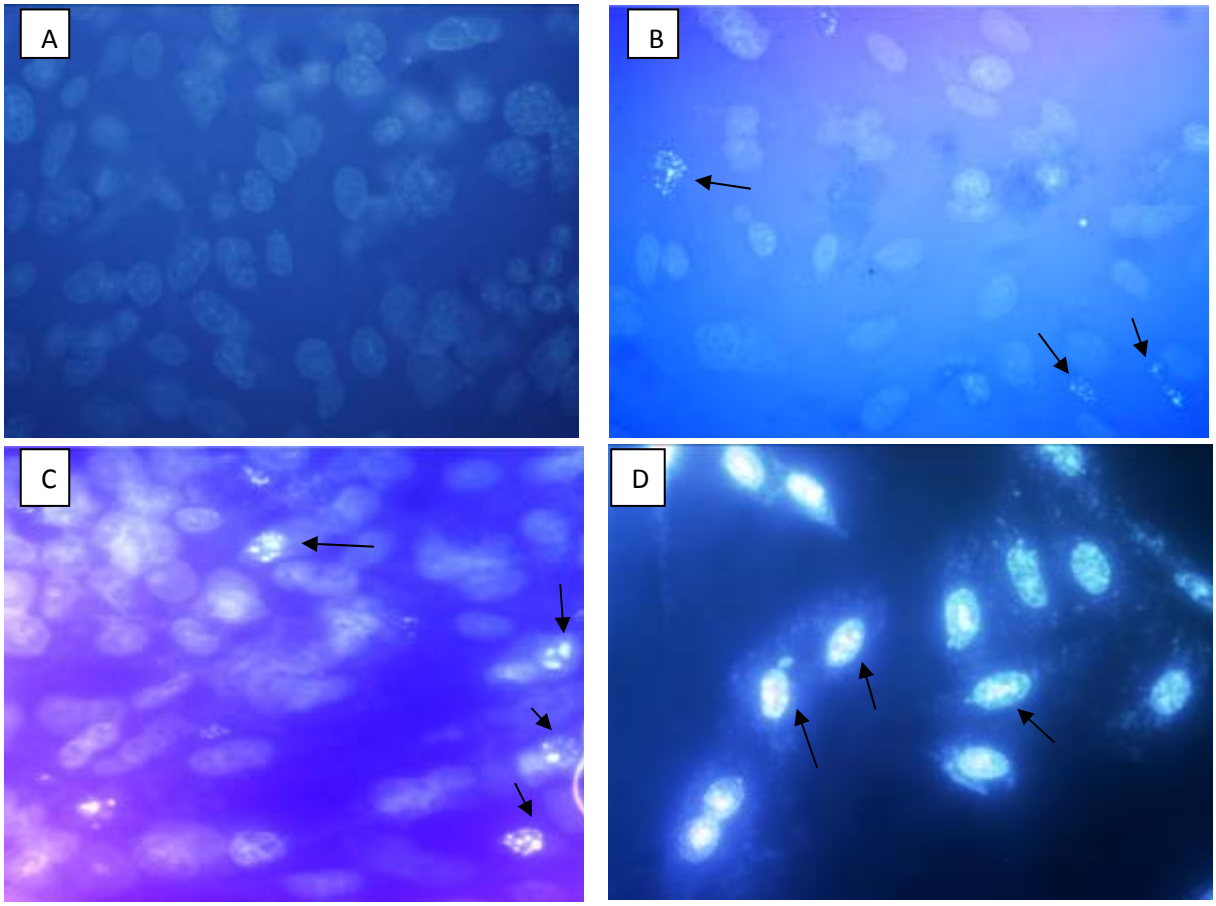


Figure 5: Chitosan induced apoptosis in HepG2 cells. Chitosan-treated (experimental group) and –untreated (control group) cells were stained with DAPI and visualized by fluorescent microscopy (magnification: x400). A (control), B (125 µg/mL) C (250 µg/mL) and D (500 µg/mL) of chitosan conducted by DAPI.

Analysis of cell cycle by flow cytometry

The fifth test we used for assay cytotoxic effects was flow cytometry analysis by PI staining. Cell cycle analysis was performed to determine cell growth inhibition. Cell cycle was divided into four stages, consisting of G1, synthesis, G2, and mitosis. According to the result of PI staining, as shown in Fig 6, the apoptotic cells in the sub-G1 area for cells which were treated with 250 µg/mL of chitosan sub-G1 peak increased remarkably, compared with the control. In addition to increasing the concentration of chitosan to 500 µg/ml, the rate of sub-G1 peak increased to 52.7%. Therefore, extracted chitosan induced apoptosis in $HepG_2$ cell line.

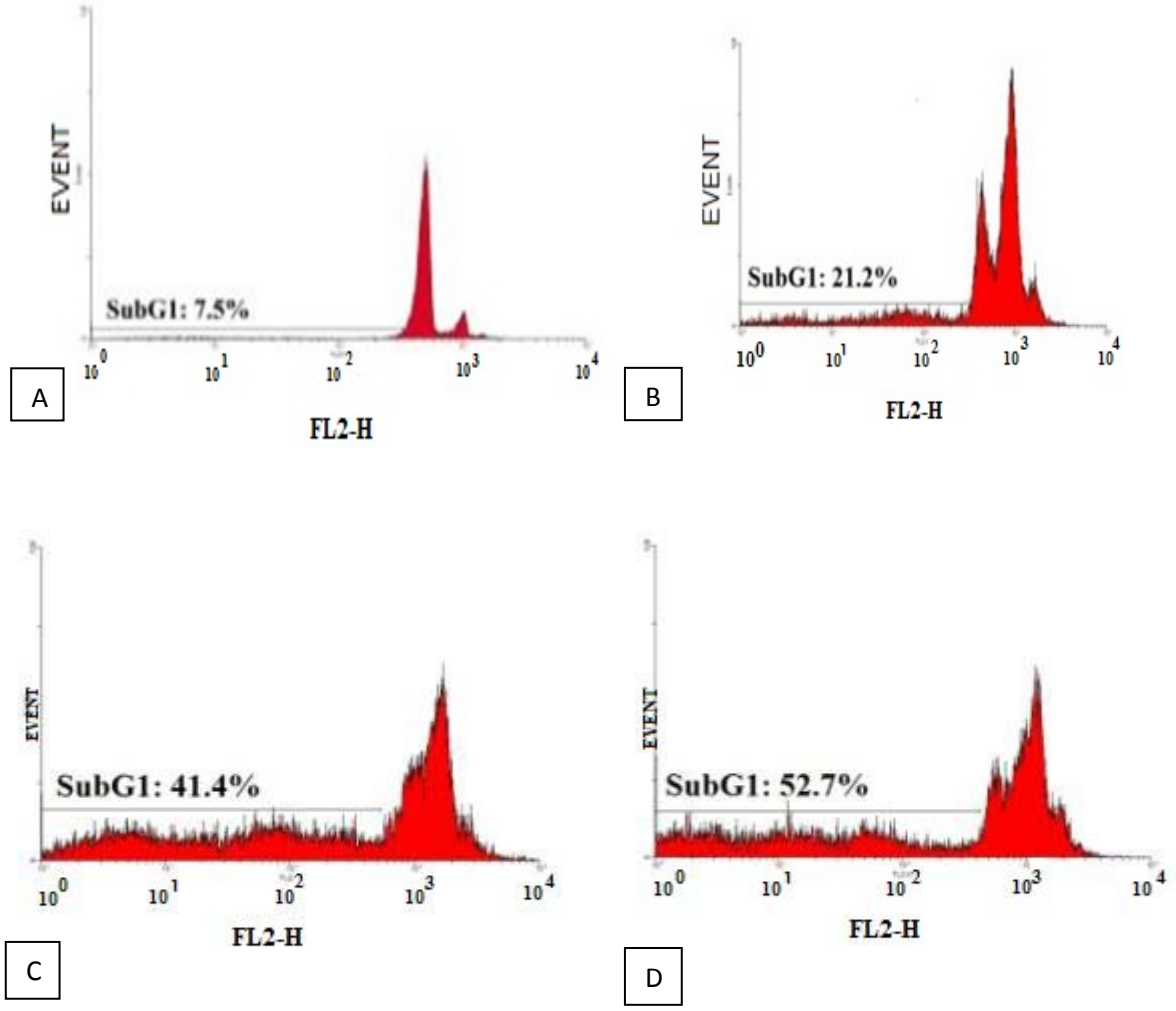
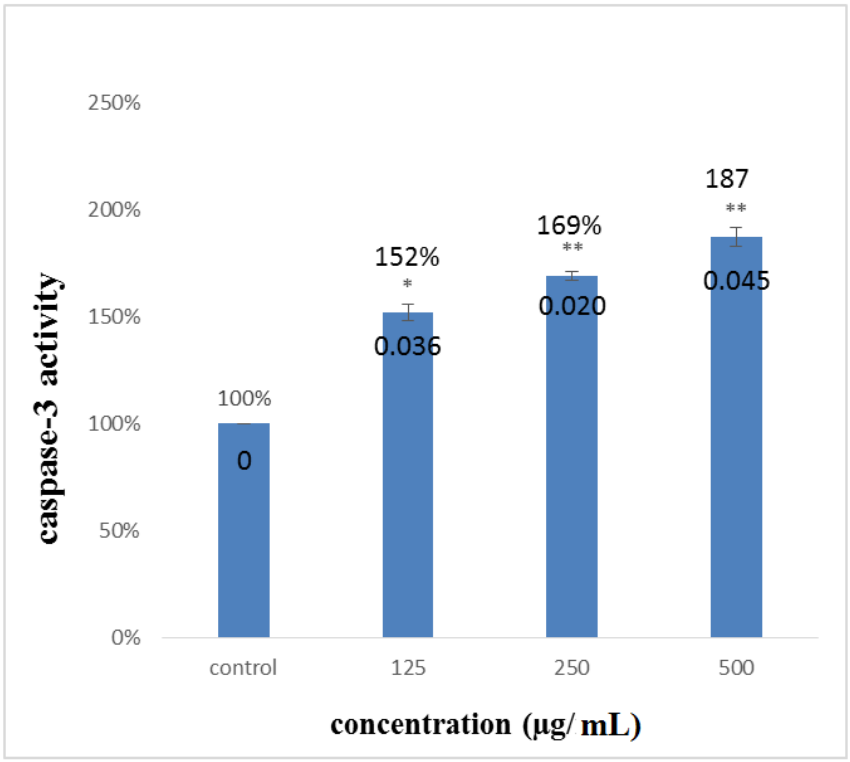


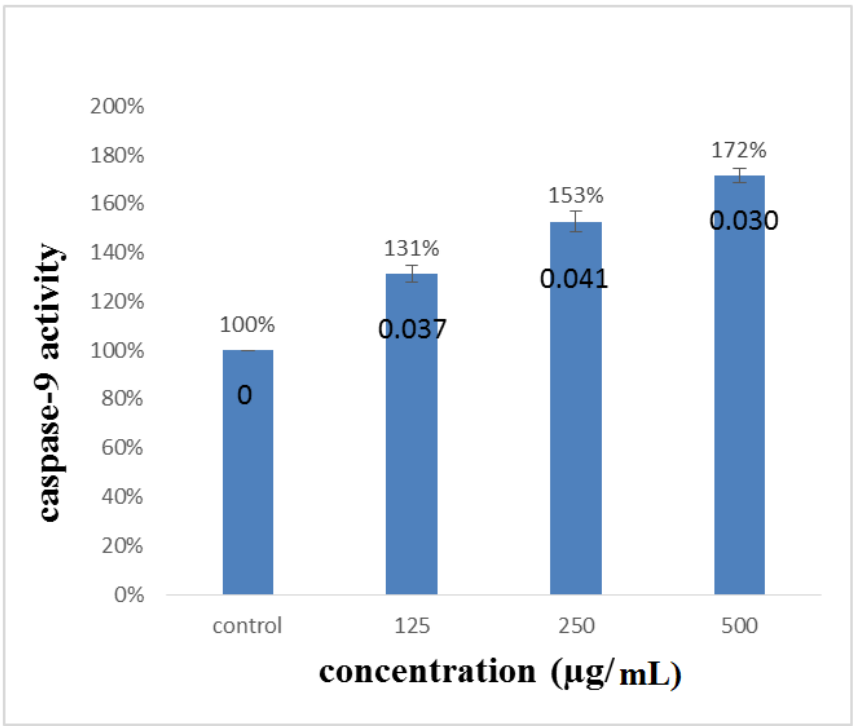
Figure 6: Induction of apoptosis on $HepG_2$ cells by extracted chitosan in vitro. Cellular apoptosis was verified by flow cytometric analysis. $HepG_2$ Cells were treated with (A) media-only, (B) 125 µg/mL, (C) 250 µg/mL, and (D) 500 µg/mL of chitosan, respectively.

Caspase activity analysis

The final test used to assay cytotoxic effects of the extracted chitosan was caspase activity analysis. The apoptotic effect of chitosan was investigated by determining caspase-3 and caspase-9 activity related to different concentrations of protein content for cells treated with 125, 250, and 500 µg/mL of chitosan. The obtained results showed that enzyme activity increased in a dose-dependent manner which was significant only for caspase-3 activity (Fig. 7).



(A)



(B)

Figure 7 (A, B): Effect of treatment with chitosan on caspase-3 and caspase-9 activity in $HepG_2$ cells. The data were represented as mean ± SD. Significant differences (p<0.05) in caspase-3, and caspase-9 activity were found between the treatment and control groups.

Table 2 (A,B): Effect of the treatment with chitosan on caspase-3 and caspase-9 activity in $HepG_2$ cells. The data were represented as mean ± SD.

Concentration(µg/mL)	Caspase-3 activity
Control	100±0
125 µg/mL	152±0.036
250 µg/mL	169±0.020
500 µg/mL	189±0.045

(A)

Concentration(µg/mL)	Caspase-9 activity
Control	100±0
125 µg/mL	131±0.037
250 µg/mL	153±0.041
500 µg/mL	172±0.030

(B)

Discussion

Production of chitosan from the existed chitin through nature is important, because natural types of chitosan may have novel usages in biological science as compared to the available commercial chitosan. To date, chitin, as a natural polymer, has been extracted from different natural resources, including crustacean exoskeleton (e.g. shrimps and crabs), insect cuticle, squid pens, and fungi cell membrane. In this research, induction of apoptosis in liver cancer cell line using chitosan extracted from Chiton shells was investigated. Some studies reported that cytotoxicity of chitosan is inversely correlated with its molecular weight. The low molecular weight of chitosan exerted stronger cytotoxic effects on all the cancer cells than the high molecular weight. For example, cytotoxic properties of chitosan against oral squamous cell carcinoma (SCC) cells varied among the cell lines tested; chitosan exerts selective toxicity to oral SCC cells and even to the opposite effects on non-cancer keratinocytes (Wimardani *et al*., 2012).

Shen *et al*. (2009) reported chitooligosaccharides extracted from fungi inhibited human hepatocellular carcinoma (HepG2) cell proliferation. According to their result, the growth rate of $HepG_2$ cells in response to 1000 µg/mL of chitooligosaccharides was 62 and 49% after 2 and 3 days, respectively (IC_{50}: 1000 µg/mL after 72 h) (Shen *et al*., 2009). Thus, the power of induce apoptosis using chitosan extracted from Chiton shell is more compared to the chitooligosaccharides extracted from fungi. In another study, Parida *et al*. (2013) reported that the commercial chitosan has cytotoxic effect (IC_{50}: 40 µg/ml) on lymphoma cancer cell lines (SUDHL-4)(Parida *et al.,* 2013). Probably, this difference is due to the purity of the commercial chitosan and chitosan extracted from Chiton shells.

Guan *et al*. (2012) reported that 250 µg/ml N-trimethyl chitosan (TMC) significantly induced apoptosis on $HepG_2$. Therefore, according to our findings, apoptotic effect of the extracted chitosan from chiton shell is in line with Guan *et*

al's. (2012) result. On the other hand, Lopez-Heras *et al*. (2014) in their research evaluated the potential of chitosan-stabilized selenium nanoparticles to induce cell cycle arrest and to inhibit *in-vitro* invasiveness in HepG2 cells. According to the result of Lopez-Heras *et al*. (2014), flow cytometry (AnnexinV/PI) confirmed that the percentage of apoptotic or necrotic cell populations in HepG2 cells exposed to Chitosan-Stabilized Selenium Nanoparticles was not significantly different from the control cells (Lopez-Heras, 2014). So, it seems that the extracted chitosan from shell of Chiton has more cytotoxic effects on HepG2 cells as compared to those of Chitosan-Stabilized Selenium Nanoparticles.

Guo *et al*. (2014) used chitosan and derivatives chitosan for anticancer effect on $HepG_2$ cells. In the obtained images from fluorescence microscopy, chromatin condensation and nuclear fragmentation are clear (Guo *et al*., 2014).

Shen *et al*. (2009) reported that chitooligosaccharides which were extracted from fungi induced cell growth inhibition in $HepG_2$ cells. $HepG_2$ cells cultured in 10% FBS medium were treated with 250 to 1000 µg/mL of chitooligosaccharides for 48 h (Shen *et al*., 2009). The result of Shen *et al*. (2009) showed that chitooligosaccharides treatment significantly inhibited the percentage of S-phase cells among the $HepG_2$ cells. In another study, Liu *et al*. (2010) reported that TMC-encapsulated camptothecin (CPT) induced apoptosis in B16-F10 mouse melanoma cell lines. After the treatment, PI staining was performed. The result showed that camptothecin-encapsulated with N-trimethyl chitosan (CPT-TMC) efficiently inhibited B16-F10 cells proliferation and increased apoptosis *in vitro* (Liu *et al*., 2010). Thus, our finding conformed more compared to that of Shen *et al*. (2009) and Liu *et al*. (2010).

Apoptosis is coordinated by a family of cysteine proteases known as caspases. The basic effectors of apoptosis in fold proteases from the caspase family were inhabited as hidden precursors in most nucleated animal cells. According to the previous study on caspase, mammalians contain fourteen caspase, such as caspase 3 and caspase 9. Identification pathways of caspase-3 activity have been performed which is relevant or irrelevant release of mitochondrial cytochrome c and caspase-9 function. Caspase-3 activation is the sign of apoptosis, and is essential for apoptotic chromatin condensation and DNA fragmentation in all the cells (Namvar *et al*., 2014). Wimardhani *et al*. (2014) reported 800 µg/mL of low molecular weight chitosan (LMWC) induced apoptosis on oral cancer cell line. The measurement of caspase 3, caspase 8, and caspase 9 activity was performed after the treatment by LMWC. LMWC increased the activities of caspase-3, caspase-8, and caspase-9, although these increases were not significant (Wimardhani *et al*., 2014). Therefore, the result of the activity of caspase-3 and caspase-9 was approximately similar to that of Wimardhani *et al*. (2014).

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