

Roe protein hydrolysate of *Alburnus tarichi* induces apoptosis in breast cancer MCF-7 and MDA-MB-231 cells through a caspase-dependent pathway

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Abstract. The protein hydrolysates of fishes have been reported to be a potential source of many health benefits components for pharmaceutical or nutritional applications. The aim of this study is to examine the possible antiproliferative function of roe protein hydrolysates of *Alburnus tarichi* using enzymatic hydrolysis against breast cancer cells and explore its detailed mechanisms. In addition, we evaluated the effects of protein hydrolysate on the proliferation and apoptosis of two human breast cancer cell lines (MCF-7 and MDA-MB-231). The cultured cells were treated with protein hydrolysate at concentrations of 0–5 µg/ml for 24 h and 48 h. Inhibition of cell proliferation, percentage of apoptotic cells, cell cycle distribution, morphological changes, DNA fragmentation, intracellular reactive oxygen species (ROS) production, and apoptotic protein levels were also examined. Decreases in proliferation of MCF-7 and MDA-MB-231 cells were observed after treatment with the protein hydrolysate in a dose-dependent manner. Distinct morphological changes, a typical pattern of fragmented DNA, and increased intracellular ROS production and apoptotic protein levels were observed in both cell lines after hydrolysate treatment ($p < 0.05$). The results suggested that the protein hydrolysate inhibits the proliferation of human breast cancer cell lines by introducing apoptosis through a caspase-dependent pathway in a dose-dependent manner.

Key words: *Alburnus tarichi* — Roe protein hydrolysate — Breast cancer — Apoptosis

Introduction

Breast cancer is an important public health problem worldwide, which is the second most common cancer diagnosed and is also a prime reason of death in women globally. It has become a fatal disease, and risk factors associated with breast cancer seem to be expanding day by day (Cedolini et al. 2014). Chemotherapy, radiation therapy, and im-

munotherapy all rely heavily on apoptosis to kill breast cancer cells (Espinosa et al. 2003). The development of chemotherapeutic agents which are unaffected by common mechanisms of chemo resistance and lack of the toxicity of predictable chemotherapeutic agents would be a foremost advance in cancer treatment (Cassidy and Misset 2002). Hence, the research and development of more effectual and less toxic drugs by the pharmaceutical industry has become indispensable, stimulating a growing interest in the identification and characterization of natural antitumor substances. The eradication of cancer in the early stages is a fundamental part of chemoprevention and measuring the cytotoxic effects of a given compound in contradiction of

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cancer cells offers useful insight into its chemoprotective potential (Lind et al. 2001).

Fishes are a huge and diverse group of animals of which more than 100 species of fishes are used as traditional medicines for tumors. Though, only a few species were analyzed by chemically and has explored. Moreover, most of the natural products obtained from fishes are assessed and developed as functional food and these varieties of products are dominated by low molecular weight compounds. The fish tissue comprises a potential source of anticancer molecules to be explored. Fish proteins exemplify an essentially untapped resource for the discovery and development of possible antitumor drugs. Isolation of effective anticancer compounds from fish tissue is a solid argument to consider that fishes might constitute a source of anticancer or chemopreventive molecules (Wang et al. 2012). Amino acids, peptides and proteins have been stated to show antitumour or antiproliferative activities. The protein hydrolysates of fishes have been reported to be a potential source of many health benefits components for pharmaceutical or nutritional applications (Hung et al. 2014). However, the peptide derived from fish protein hydrolysates has been rarely studied for antiproliferative activity.

Alburnus tarichi (pearl mullet) is an anadromic endemic carp species that migrates to streams (between mid-April and mid-July) living in the high pH waters of Lake Van and pouring into the lake for breeding. According to TurkStat data, this fish is hunted around 10,000 tons *per* year on average. Therefore, it is an economically important species. *A. tarichi* can leave approximately 6,000–16,000 eggs depending on the internal and external factors such as age, size and nutrition level of the fish. The eggs are very small and about 1 mm in diameter. Due to its fast growth and suitable price, *A. tarichi* currently is regarded a high economic value fish species in Turkey (Oğuz 2018).

To the best of our knowledge, there is no report regarding anticancer activity of fish protein hydrolysate of *A. tarichi* available. Therefore, the main objective of this study is to examine the possible antiproliferative function of roe protein hydrolysates of *A. tarichi* using enzymatic hydrolysis against breast cancer cells and explore its detailed mechanisms.

Materials and Methods

Preparation of defatted fish roe

A. tarichi was obtained freshly from several fishermen in Van, Turkey, in March 2019. In order to carry out the study, necessary permissions were obtained from Van Animal Experiments Local Ethics Committee of Van Yüzüncü Yil University (Decision number: 2019/03, Date:

28.03.2019). The fishes were placed in ice and transported to the laboratory within 1 h. All the fishes were sacrificed and the whole roes were cleaned using cool water (+4°C) and homogenized in a cool room. The homogenized roes were then freeze-dried. Afterward, lipids of the dried egg powders were extracted as described previously (Bligh and Dyer 1959). In brief, each 100 g freeze-dried homogenized roes were added to 300 ml hexane for 2 h fat extraction. This procedure was repeated three times. Moreover, the solvents were evaporated by vacuum concentration. The defatted roe protein samples were then freeze-dried. The defatted roe powders were kept in sealed polyethylene bag and then placed at –40°C until use.

Preparation of roe protein hydrolysate of *A. tarichi*

Roe protein hydrolysate was prepared from defatted *A. tarichi* roe powder using Protease N (Amano Pharmaceutical Co., Nagoya, Japan). 5 g defatted sample was suspended in a 250 ml of pH 8.0 phosphate buffer. The hydrolysis reaction was initiated by the ratio of Protease N/egg sample at 1:100 (w/w solid matter). The reaction was conducted at pH 8.0 and 50°C for 9 h. The enzymatic hydrolysis was ended by heating the mixtures at 90°C for 10 min to inactivate the protease activity. The solution containing hydrolysate was centrifuged at 5000 × *g* for 10 min at 4°C. Then, the supernatants were desalted and lyophilized to dried roe protein hydrolysate for storage at –40°C (Bligh and Dyer 1959).

Cell cultures

The human breast cancer cell lines (MCF-7 and MDA-MB-231) were selected as breast cancer cell models in this research. MCF-7 and MDA-MB-231 cell lines were obtained from American Type Culture Collection (ATCC). MCF-7 cells are useful for *in vitro* breast cancer studies due to their capacity to process estrogen *via* estrogen receptors in the cell cytoplasm, which makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line. MDA-MB-231 breast cancer cells have an epithelial-like morphology, which appear phenotypically as spindle shaped cells. The difference between the two cell lines is that MCF-7 is an ER-positive breast cancer line derived from an *in situ* carcinoma, meaning that the cancerous cells had not yet invaded surrounding tissues; however, the MDA-MB-231 is an ER-negative breast cancer line derived from a metastatic carcinoma. Selection of both cell lines helps in better illustrating and understanding the mechanism of roe protein hydrolysate induced growth inhibition on human breast cancer. Both cell lines were grown adherently as a monolayer in 75 ml plastic flasks in DMEM medium supplemented with 10% FBS (heat inactivated 30 minutes, 56°C before use), 100 U/ml penicillin,

and 100 µg/ml streptomycin in incubator under standard cultured condition (37°C and 5% CO₂). For enumeration, 30 µl of trypan blue (0.2%) stained the same volume (30 µl) of cell concentration, and neobar lam was used for counting and viability (more than 95% for adhering cell lines before testing) of the cells.

Antiproliferation assay

Cell proliferation was investigated using MTT assay (Motomura et al. 2008). Cells were detached from the 25 cm³ tissue culture flask when it achieved 80% confluency. The detached cells were pelleted by centrifugation (200 × g; 5 min). Cells (3 × 10⁴ cells/ml) were seeded onto a 96-well microtiter plate. The cells were incubated at 37°C carbon dioxide (CO₂) incubator for 24 h to give adherent cells. The test hydrolysates (0.25, 0.5, 0.75, 1, 2.5, and 5 µg/ml) were added onto the 96-well microtiter plate containing adherent cells. The untreated cells were incubated in 10% media containing 0.5% dimethyl sulfoxide (DMSO) (without addition of any test hydrolysates) as control group. The plates were incubated for 24 h, and 48 h at 37°C in a 5% CO₂ incubator. After these periods, the media were removed and 100 µl of fresh medium and 20 µl of MTT (Sigma, filter sterile, 5 mg/ml) were added to each well and further incubated for 4 h (37°C) after which the media were substituted with 150 µl DMSO. The 96-well microtiter plates were then agitated at room temperature onto an incubator shaker to dissolve the formazan crystals. The absorbance of the content of the plates was measured at 540 nm using a microplate reader. The percentage of inhibition of each test sample was calculated according to the following formula: Percentage of inhibition (%) = $(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100\%$.

The average of three replicates was then obtained. The half maximal inhibitory concentration (IC₅₀) of hydrolysate for both period was calculated using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, California, USA). Concentrations around and above the IC₅₀ value would be used in the further studies.

Morphological changes

MCF-7 and MDA-MB-231 cells were trypsinized and 5 ml of growth medium were added, then the cell suspension was mixed well. Then 2 ml of cell suspension was added to the sterilized test tubes containing cover slip. The test tubes were placed in a slanting position and kept in CO₂ incubator for 2 days. The monolayer of cells formed in the cover slip was observed under Inverted microscope (Euromex, ED Arnhem, Netherlands) and were photographed using a digital camera (Nikon, Tokyo, Japan) (Allen et al. 1997; Zhu and Wang 1997; Akter et al. 2012).

DNA fragmentation

MCF-7 and MDA-MB-231 cells (2 × 10⁵ cell/ml) were treated with 1, 2.5, and 5 µg/ml doses of roe protein hydrolysate incubated for 48 h. After the incubation period, DNA fragmentation was analyzed as described by Mendis et al. (2015). Gel electrophoresis was carried out with extracted DNA and visualized under ultraviolet (UV) light to assess the effect on DNA fragmentation using a Mini BisPro gel imaging system (DNR Bioimaging Systems, Jerusalem, Israel) following electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Cell cycle distribution

We performed flow cytometric analysis to assess whether roe protein hydrolysate affected cell cycle progression in MCF-7 and MDA-MB-231 cells. Being treated with different concentrations of roe protein hydrolysate (1, 2.5, and 5 µg/ml) for 48 h, the cells were collected and fixed with 70% ice-cold ethanol. Thereafter we stored the cells at -20°C. Then the cells were washed and resuspended in cold PBS. RNA was digested with RNase A (100 µg/ml) and DNA was stained with propidium iodide (PI) (50 µg/ml). After incubation for 30 min in the dark at 37°C, flow cytometry was performed to analyze cell cycle distribution (FACSCalibur, Becton Dickinson, USA) (Zhen et al. 2014).

Apoptosis by annexin V/PI

We conducted the annexin V-FITC and PI staining to evaluate the effects of roe protein hydrolysate (1, 2.5, and 5 µg/ml) on apoptosis in MCF-7 and MDA-MB-231 cells. Firstly, MCF-7 and MDA-MB-231 cells were plated into 6-well plates (Corning, USA) and then incubated with roe protein hydrolysate in different concentrations for 48 h. The cells were collected, centrifuged, and then incubated with Annexin V-FITC/PI for 15 min at room temperature in the dark and was finally analyzed flow-cytometrically (Pilane et al. 2015).

Intracellular ROS

The intracellular reactive oxygen species (ROS) was estimated by fluorescent probe, 2',7'-dichlorohydrofluorescein diacetate (H₂-DCF-DA). This dye is deacetylated by intracellular esterase and converted to nonfluorescent 2',7'-dichlorohydrofluorescein (H₂-DCF), which is rapidly oxidized to the highly fluorescent compound 2',7'-dichlorohydrofluorescein (DCF) in the presence of ROS. The MCF-7 and MDA-MB-231 cells were dispensed into 24-well plates for 1 h. Then, the cells were incubated only with the DMEM medium used as control or treated with roe protein hydrolysate

(1, 2.5, and 5 $\mu\text{g/ml}$) at 37°C for 22 h. After 22 h of exposure, the samples were removed and centrifuged at $200 \times g$ for 5 min, washed with PBS at 37°C, and labeled with $\text{H}_2\text{-DCFH-DA}$ for 30 min in the dark conditions, at 37°C. Then, cells were washed with phosphate buffered saline (PBS) and ROS formation was quantified by flow cytometer (FACSCalibur, Becton Dickinson, USA) at 488 nm excitation and 532 emission considering a minimum of 10,000 total events for each sample in FlowJo program as described by Wagner et al. (2010). Data are presented as fluorescence intensity (a.u.).

Western blot analysis

MCF-7 and MDA-MB-231 cells were treated with various concentrations of roe protein hydrolysate (1, 2.5, and 5 $\mu\text{g/ml}$) for 48 h; then MCF-7 and MDA-MB-231 cells were lysed in lysis buffer (Radioimmunoprecipitation assay buffer (RIPA), 1 mM phenylmethylsulfonyl fluoride (PMSF)) and phosphatase inhibitors (Roche, Germany) to extract the total protein. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8%, 12% gels) under reducing conditions. The proteins were then electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed

milk and incubated with anti-Caspase-3, anti-Caspase-7, anti-Caspase-8, anti-Caspase-9, anti-TNF- α , anti-Bcl-2, anti-Bax, anti-COX-2, anti-p53, anti-Fas, anti-ErbB2, and anti- β -actin antibodies, respectively (1:1000; Cell Signaling Technology) overnight at 4°C. After that, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h and then, treated with an enhanced chemiluminescence (ECL) substrate kit (Thermo Fisher Scientific Inc., Massachusetts, USA). Protein bands were quantified by ImageJ software with β -actin as internal loading controls and for normalizing purpose.

Statistical analysis

All experiments were performed in triplicate and results were presented as mean \pm standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) plus Tukey *post-hoc* analysis. Statistical analyses were performed using the (Statistical Package for the Social Sciences) SPSS software version 15.0 (SPSS Inc., Illinois, USA). A value $p < 0.05$ was considered statistically significant.

Results

To determine the growth inhibitions of the protein hydrolysate on human breast cancer cell lines, the MTT assay was used to evaluate the antiproliferative activities after protein hydrolysate (0.25–5 $\mu\text{g/ml}$) treatment for 24 h and 48 h. Figure 1A and B shows that the protein hydrolysate inhibited the growth of both cell lines in a dose-dependent manner compared to control. The maximum growth inhibitions were achieved after 48-h treatment with protein hydrolysate at the concentration of 5 $\mu\text{g/ml}$ on MCF-7 (87.6%) (Fig. 1A) and MDA-MB-231 (89.1%; Fig. 1B). On the other hand, the growth inhibitions were achieved as 86.5% and 88.2%, at the end of the 24-h incubation with protein hydrolysate at the concentration of 5 $\mu\text{g/ml}$ on MCF-7 and MDA-MB-231 cell lines, respectively (Fig. 1A and B). Significant decreases in survival of protein hydrolysate treated MCF-7 and MDA-MB-231 cells were observed compared to Control group ($p < 0.05$). The antiproliferative activities of protein hydrolysate on both breast cancer cell lines were increased along with the increasing dosages of protein hydrolysate. There were no significant differences in growth inhibitions of protein hydrolysate between the dosages of 5 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ on MCF-7 and MDA-MB-231 cells ($p > 0.05$). These results suggest that the growth inhibition activity of protein hydrolysate was saturated at a concentration of 2.5 $\mu\text{g/ml}$ on MCF-7 and MDA-MB-231 cells. The protein hydrolysate had shown relatively higher antiproliferative activities on MDA-MB-231 cells at each concentration than on MCF-7 cells, but these differences were not statistically

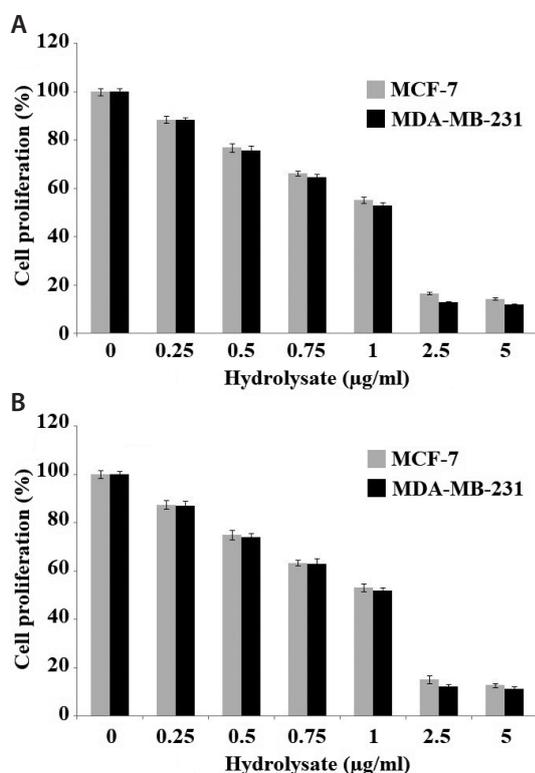


Figure 1. Dose-dependent anticancer effects of roe protein hydrolysate of *A. tarichi* on cell proliferation of MCF-7 and MDA-MB-231 cell lines for 24 h (A) and for 48 h (B) incubations.

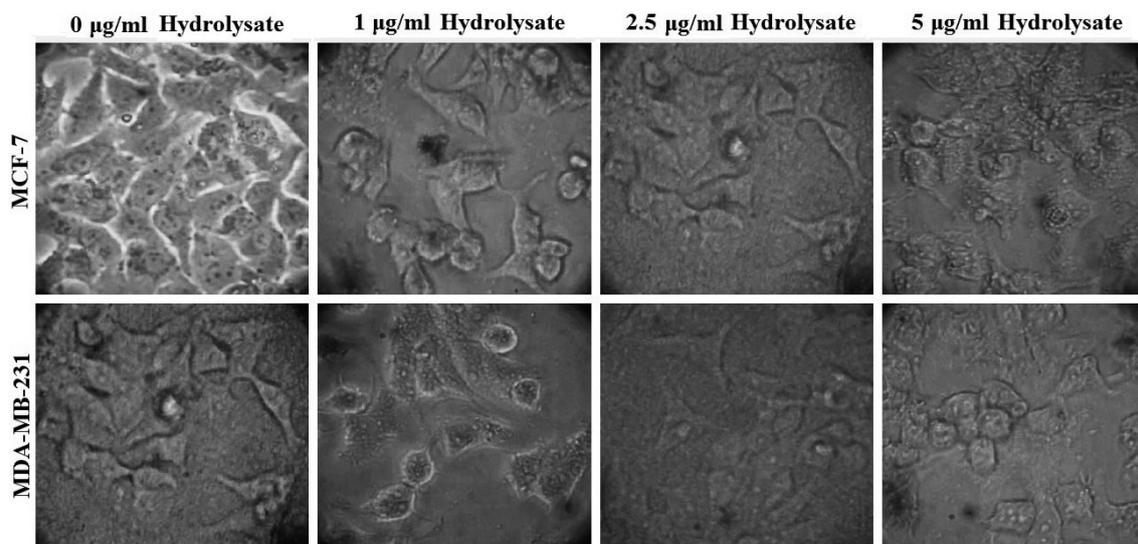


Figure 2. Effect of treatment with different concentration of roe protein hydrolysate on the morphology of MCF-7 and MDA-MB-231 cells. Cells were visualized using an inverted microscope (Euromex, ED Arnhem, Netherlands). Magnification, 100 \times .

significant ($p > 0.05$) (Fig. 1A and B). IC_{50} values of roe protein hydrolysate on MCF-7 and MDA-MB-231 cells for 48 h incubation were found as 1.89 $\mu\text{g/ml}$ and 1.81 $\mu\text{g/ml}$, respectively. Concentrations around and above the IC_{50} value of the protein hydrolysate (1, 2.5, and 5 $\mu\text{g/ml}$) were used in the following studies.

The morphological changes of the cells were visualized by inverted microscope. The control MCF-7 and MDA-MB-231 cells which were not treated with samples did not show any morphological changes. But the treated cell lines exhibited irregular confluent aggregates with rounded and polygonal cell morphology, this phenomenon was observed more frequently with increased concentrations of the roe protein hydrolysate (Fig. 2).

DNA fragmentation is a classic indicator of apoptosis. In the present study, MCF-7 and MDA-MB-231 cells treated with roe protein hydrolysate showed a DNA fragmentation in a time- and dose-dependent manner with both high molecular weight DNA and smaller DNA fragments extending from 2 kbp to 100 bp. The control treated with 0.1% DMSO showed clear bands of intact DNA (Fig. 3).

To examine whether roe protein hydrolysate-induced growth inhibition was associated with cell cycle regulation, the cell cycle distribution was analyzed by flow cytometry (Fig. 4). After MCF-7 and MDA-MB-231 cells were incubated with the hydrolysate for 48 h, cells were harvested and further prepared for cell cycle analysis. Figure 4A (MCF-7 cells) and Figure 4B (MDA-MB-231 cells) showed that cells accumulated in the S phase of the cell cycle after the hydrolysate treatment, whereas the percentage of cells in G_0/G_1 phase reduced significantly. Especially, the percentage of

cells in S phase increased by 29.4% and 30.3% for MCF-7 and MDA-MB-231 cells, respectively, when the hydrolysate concentration was 5 $\mu\text{g/ml}$, compared to that of the untreated control ($p < 0.05$) (Fig. 4A and B).

Induction of apoptosis by roe protein hydrolysate was quantitatively determined by flow-cytometric analysis. For MCF-7 cells, early apoptotic cells increased from 11.1% at 2.5 $\mu\text{g/ml}$ to 14.3% at 5 $\mu\text{g/ml}$ of roe protein hydrolysate compared to 4.9% in the control at 48 h postincubation (Fig. 5A) ($p < 0.05$). For MDA-MB-231 cells, early apoptotic

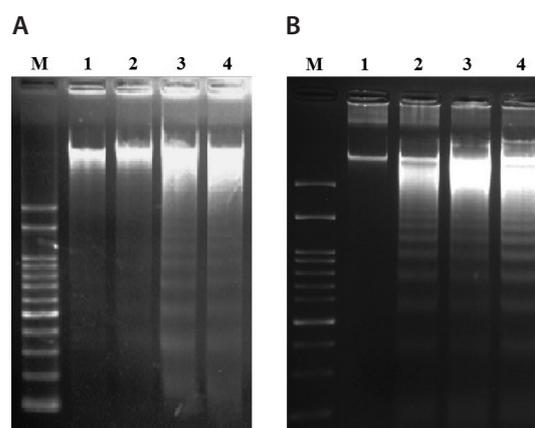


Figure 3. Induction of DNA fragmentation in MCF-7 cells (A) and MDA-MB-231 cells (B) by different concentration of roe protein hydrolysate. M, marker; Line 1, 0 $\mu\text{g/ml}$ hydrolysate; Line 2, 1 $\mu\text{g/ml}$ hydrolysate; Line 3, 2.5 $\mu\text{g/ml}$ hydrolysate; Line 4, 5 $\mu\text{g/ml}$ hydrolysate.

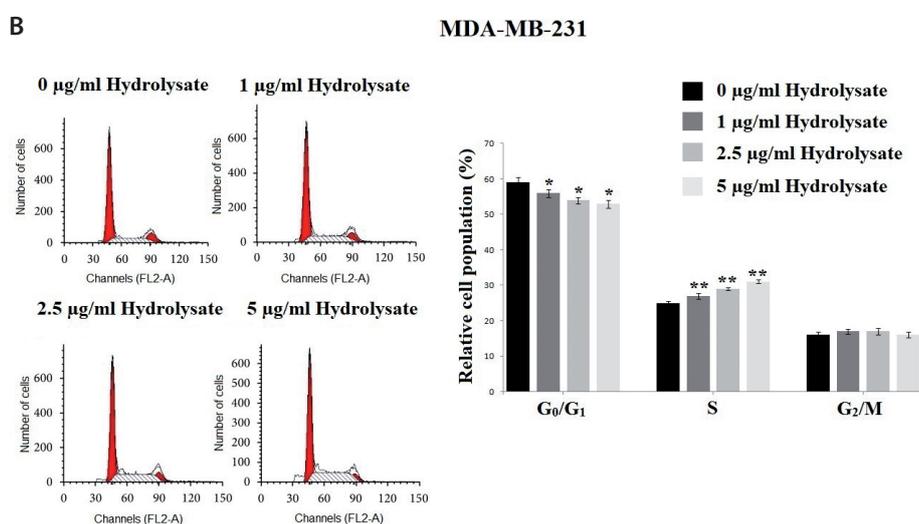
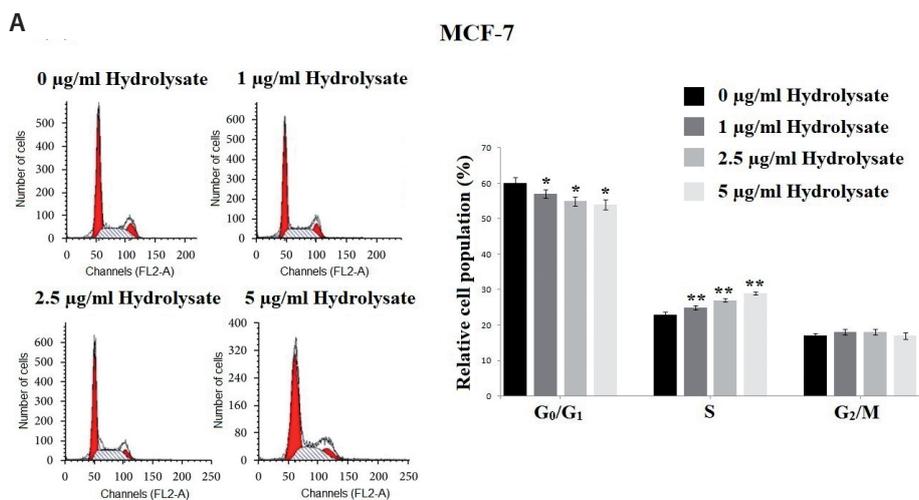


Figure 4. Effects of roe protein hydrolysate on the cell cycle. The cell cycle distributions in MCF-7 cells (A) and MDA-MB-231 cells (B) were determined by propidium iodide staining and flow cytometry analysis after being treated with different concentrations of roe protein hydrolysate (0, 1, 2.5, and 5 µg/ml) for 48 h. The ratios are expressed as the mean ratios ± SD of three independent experiments. * statistically significantly lower than 0 µg/ml hydrolysate-treated group ($p < 0.05$); ** statistically significantly higher than 0 µg/ml hydrolysate-treated group ($p < 0.05$).

cells were found to be 0.5% in the Control group, 0.1% at 2.5 µg/ml, and 0.2% at 5 µg/ml roe protein hydrolysate-treated groups (Fig. 5B) ($p > 0.05$). The number of late apoptotic cells increased to 47.2% at 2.5 µg/ml and 50.2% at 5 µg/ml of protein hydrolysate for MCF-7 cells ($p < 0.05$) (Fig. 5A), and 38.1% at 2.5 µg/ml and 48.1% at 5 µg/ml of protein hydrolysate for MDA-MB-231 cells ($p < 0.05$) (Fig. 5B).

Upgrading in the level of ROS or/and reduction in the level of antioxidants can trigger mitochondrial-initiated events leading to apoptosis. Furthermore, production of ROS can disrupt the homeostasis in the enzyme system of ROS scavenging antioxidants. Generation of ROS was measured in the treated MCF-7 and MDA-MB-231 cells with different concentrations of roe protein hydrolysate. In our study, exposure to hydrolysate caused the significant production of intracellular ROS in dose-dependent

manner in the treated MCF-7 and MDA-MB-231 cells ($p < 0.05$) (Fig. 6).

Significantly increased expressions of caspase-3 and caspase-7 were detected in both cell lines in the presence of roe protein hydrolysate (5 µg/ml) at 48 h ($p < 0.05$). Roe protein hydrolysate at the concentration of 2.5 and 5 µg/ml induced significant increases in relative caspase-8 and caspase-9 intensity in MCF-7 and MDA-MB-231 cells compared to the untreated cells ($p < 0.05$) (Fig. 7A). Significantly increased expression of p53 were observed after 48 h of incubation with roe protein hydrolysate in both cell lines ($p < 0.05$). However, the hydrolysate showed significant down-regulation on COX-2 levels in MCF-7 cells ($p < 0.05$) while no significant decreased levels of COX-2 in MDA-MB-231 were observed ($p > 0.05$) (Fig. 7B). TNF-α and Fas protein expressions increased significantly after hydrolysate treatment compared to control group in a dose-dependent manner in both cell lines

($p < 0.05$) (Fig. 7B). Significant increases in Bax expression and decreases in Bcl-2 levels were observed in hydrolysate treated MCF-7 and MDA-MB-231 cells after 48 h compared to the untreated cells ($p < 0.05$) (Fig. 7C). On the other hand, the hydrolysate lead to significant ($p < 0.05$) decreases in the expression of ErbB-2 in both cell lines (Fig. 7C).

Discussion

The protein hydrolysates of fishes have been reported to be a potential source for anticancer activity. It has been evidenced that some peptides isolated from fish tissues exhibit

anticancer activity, inhibit the cell proliferation and also have cytotoxic effect against tumor cells (Shahidi and Zhong 2008; Hsu et al. 2009). In the present study, protein hydrolysate of *A. tarichi* roe showed dose-dependent anticancer activity on MCF-7 and MDA-MB-231 cells. Furthermore, protein hydrolysate had shown relatively higher antiproliferative activities on MCF-7 cells at each concentration than on MDA-MB-231 cells. The reason of this situation is probably because MDA-MB-231 is more resistant to anticancer treatment since it is derived from a cancerous site that was in metastatic status (Cedolini et al. 2014). Similarly Picot et al. (2006) recognised that protein hydrolysates of three cod, three plaice, three blue whiting and one salmon species acted

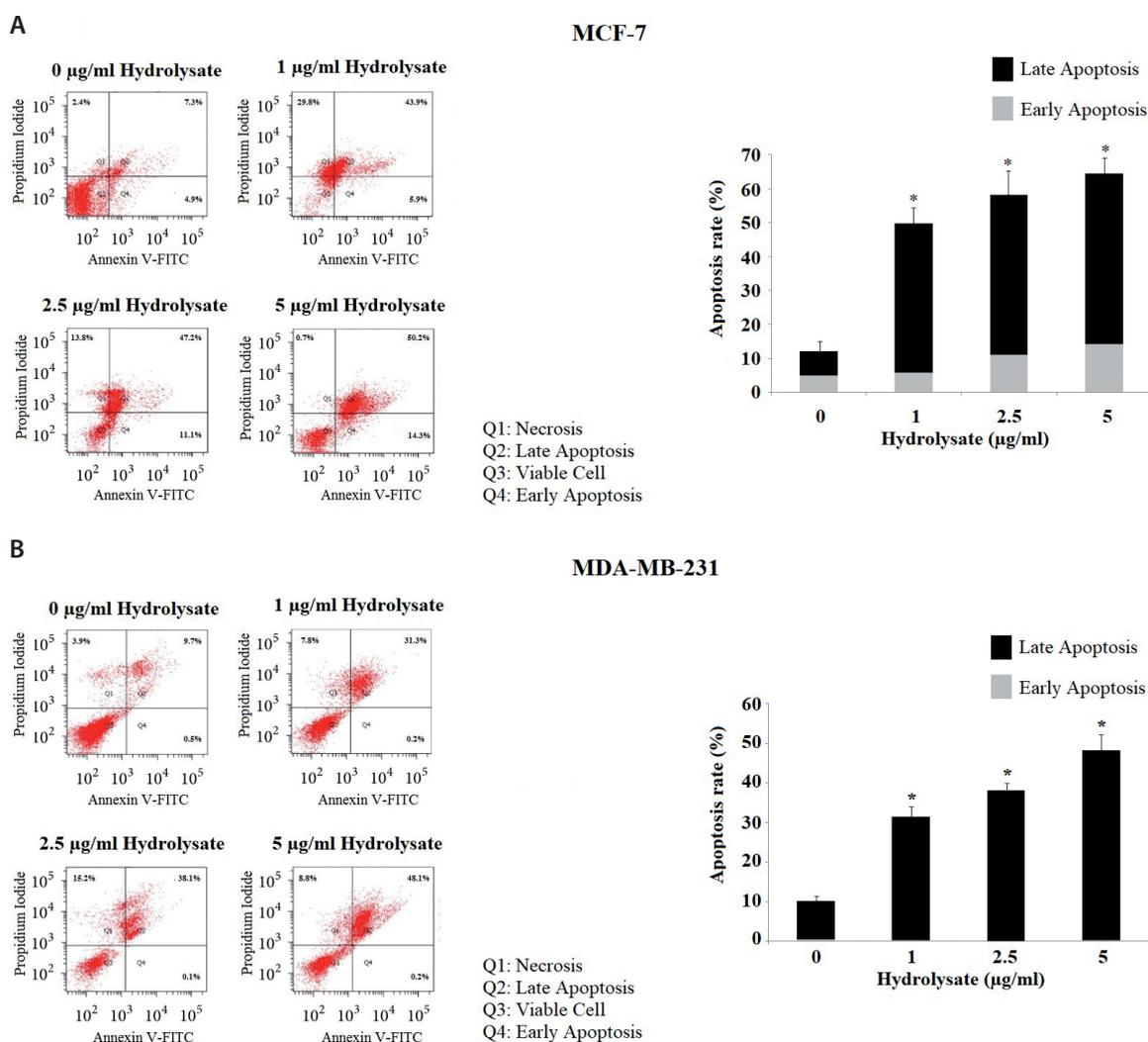


Figure 5. Flow-cytometry analysis of roe protein hydrolysate-induced apoptosis in MCF-7 cells (A) and MDA-MB-231 cells (B). The cells were treated with the indicated concentrations of hydrolysate for 48 h, followed by labeling for phosphatidylserine externalization with FITC-annexin-V and cell membrane integrity with PI. Apoptotic staining profiles were analysed using flow cytometry. Independent experiments were performed in triplicate. Data are expressed as mean \pm SD. FITC, fluorescein isothiocyanate; PI, propidium iodide. * statistically significantly higher than 0 $\mu\text{g/ml}$ hydrolysate-treated group ($p < 0.05$).

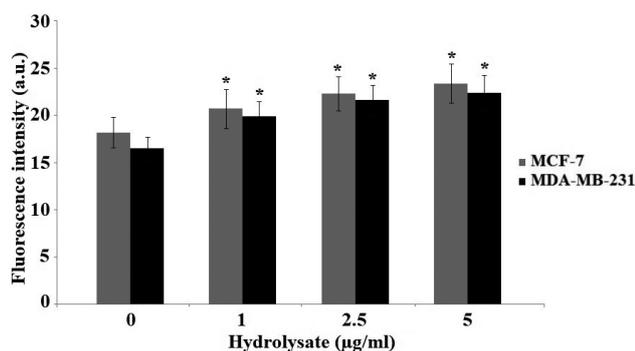


Figure 6. The effect of roe protein hydrolysate treatment on ROS generation in MCF-7 and MDA-MB-231 cells. Independent experiments were performed in triplicate. Data are expressed as mean \pm SD. ROS, reactive oxygen species. * statistically significantly higher than 0 $\mu\text{g/ml}$ hydrolysate-treated group ($p < 0.05$).

as significant inhibitors against MCF-7/6 and MDA-MB-231 human breast carcinoma cell lines. Alemán et al. (2011) also reported anticancer activity of esperase and alcalase hydrolysed squid gelatin hydrolysates on MCF-7 cells. In addition Hsu et al. (2011) also documented anticancer activity of papain and protease hydrolysates of tuna dark muscle on MCF-7 cells in dose-dependent manner.

The IC_{50} value of the present study was comparably lower than the reported value of Naqash and Nazeer (2012) for *Nemipterus japonicus* (48.5 $\mu\text{g/ml}$) and *Exocoetus volitans* (21.6 $\mu\text{g/ml}$) muscle protein hydrolysates in liver carcinoma cell line (Hep-G2). Further, Alemán et al. (2011) reported the IC_{50} values of esperase and alcalase squid gelatin hydrolysates on MCF-7 cells at 24 h as 0.15 mg/ml and 1.18 mg/ml, respectively. In addition, Hsu et al. (2011) also recorded the IC_{50} values of papain and protease hydrolysates of tuna dark muscle on MCF-7 cells as 8.1 μM and 8.8 μM , respectively.

DNA fragmentation is a typical indicator of apoptosis but not with the other mode of cell death like necrosis. The mechanism of action of several anticancer drugs is based on their capacity to induce apoptosis (Motomura et al. 2008). In the present study, DNA fragmentation assay was observed for displaying the apoptotic reaction. Lee et al. (2004) studied anticancer activity of peptide isolated from anchovy sauce, and their capabilities to induce apoptosis in U937 human lymphoma cell and revealed that hydrophobic peptide was found to have strong antiproliferative activity against U937 cell by inducing apoptosis.

The morphological changes of the cells treated with protein hydrolysate of *A. tarichi* roe were visualized. The results revealed that the untreated control MCF-7 and MDA-MB-231 cells did not show any morphological changes. But the treated cells were shown irregular confluent aggregates with rounded and polygonal cell morphology and which was found to be more with increasing the concentrations

of protein hydrolysate. Similarly, Wang et al. (2012) also reported morphological changes in the A549 cells treated with Syngnathusin, a novel peptide isolated from *Syngnathus acus*.

The majority of anti-cancer studies have reported perturbation of the S/G₂ phase transition with a decrease of cells in the G₀/G₁ phase of the cell cycle and an increase of cells in the S phase (Lin et al. 2013). In the present study, the percentage proportion was increased in the S phase cells and reduced in the G₀/G₁ phase cells following roe protein hydrolysate treatment in a dose-dependent manner, indicating that the inhibitory effect of roe protein hydrolysate extract on MCF-7 and MDA-MB-231 cell proliferation is mediated by S phase cell cycle arrest.

To our knowledge, the inhibition of cell proliferation is involved in apoptosis and block of cell cycle progression. It has been reported that sea squirt (*Halocynthia roretzi*) hydrolysate induced HT-29 colon cancer cells apoptosis via the increased expression of p53 and the modulation of Bcl-2/Bax protein expression (Oh et al. 2019). Hung et al. (2014) reported that protein hydrolysates from tuna cooking juice induced MCF-7 cells apoptosis by the detection of flow cytometry. Similar to these results, protein hydrolysate of *A. tarichi* roe induces the onset of apoptosis on MCF-7 and MDA-MB-231 cells in the current study.

ROS plays a key role in various biochemical processes like apoptosis and cell proliferation. However, high levels of ROS can also cause cellular damage, depending on the levels and duration of ROS stress (Pelicano et al. 2004). Hence, by exploiting these dose-dependent ROS generation, we can trigger cell death by using exogenous ROS-generating agents. In our study, protein hydrolysate of *A. tarichi* roe was found to increase the ROS generation on MCF-7 and MDA-MB-231 cells. Hence, protein hydrolysate of *A. tarichi* roe may be overlooked as a potential exogenous candidate generating ROS to induce apoptosis in breast cancer cells.

The activation of caspase-3 and caspase-7 were observed in both cell lines after treatment with protein hydrolysate which is consistent with the resulting morphological changes and the DNA fragmentation (Kumar 1999). Therefore, the results suggested that protein hydrolysate-induced MCF-7 and MDA-MB-231 cells death was involved in a mechanism of caspase-dependent apoptosis. On the other hand, protein hydrolysate treatment increased caspase-8 and caspase-9 expressions and induced apoptosis in MCF-7 and MDA-MB-231 cells. These results suggest that protein hydrolysate triggers apoptosis via a combination of intrinsic and extrinsic pathways.

Studies have suggested that the up-regulation of the p53 protein is in accordance with growth inhibition of cancerous cells when exposed to various anti-cancer agents (Sun 2006; Comşa et al. 2015; Engeland 2018). In our study, the expression of p53 increased significantly in protein hydrolysate-treated MCF-7 and MDA-MB-231 cells and suggests that

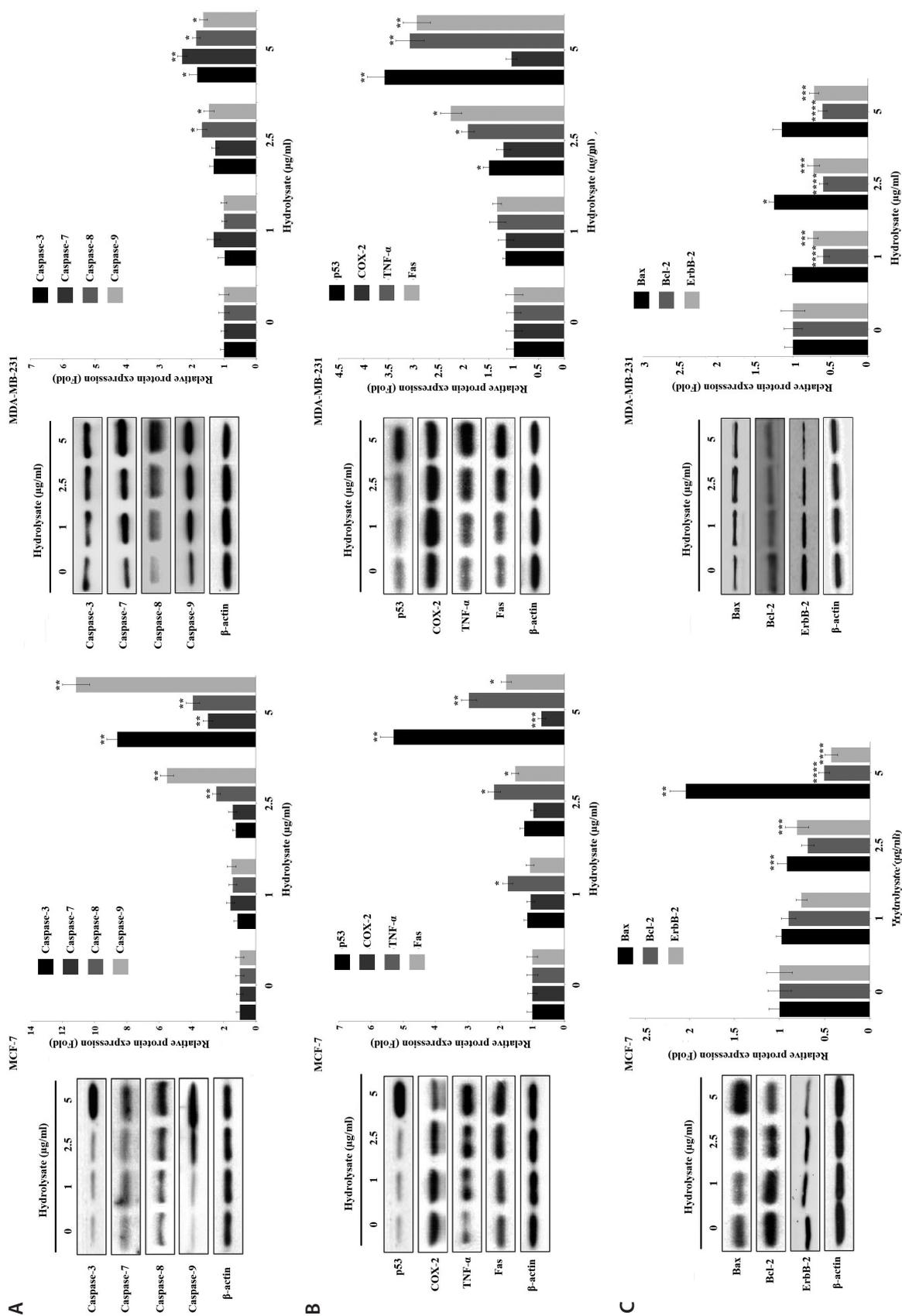


Figure 7. Effects of roe protein hydrolysate on the expressions of caspase proteins (A), p53, COX-2, TNF-α, and Fas proteins (B), and Bax, Bcl-2, and ErbB-2 proteins (C) in MCF-7 and MDA-MB-231 cells, respectively. Independent experiments were performed in triplicate. Data are expressed as mean ± SD. COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor alpha; Bcl-2, B-cell lymphoma 2 protein; Bax, Bcl-2-associated X protein. **** statistically significantly higher than 0 μg/ml hydrolysate-treated group ($p < 0.05$, $p < 0.001$, respectively); **** statistically significantly lower than 0 μg/ml hydrolysate-treated group ($p < 0.05$, $p < 0.001$, respectively).

protein hydrolysate up-regulates the activated p53 which may either trigger the onset of DNA repair or induces the apoptosis via mitochondrial-dependent pathway by regulating the expressions of downstream molecules such as Bax, Bcl-2, and Fas. COX-2 over-expression may result in the suppression of proapoptotic proteins such as Bax and Bcl-xL during apoptosis (Sarkar et al. 2007; Thill et al. 2014; Regulski et al. 2016). In our research, the protein hydrolysate displayed a down-regulating effect on COX-2 expression in MCF-7 cells; while the COX-2 expression remained unchanged in a MDA-MB-231 cell line. These results suggested that protein hydrolysate suppress the expression of COX-2 which leads to the cell growth inhibition *via* decreasing of estrogen levels in an ER-positive MCF-7 cell line; however, protein hydrolysate has no effect on regulating COX-2 expression in an ER-negative MDA-MB-231 cell line. This result is agree with the previous study by Harris et al. (1999), who suggested that PGs may also stimulate proliferation indirectly *via* increased estrogen biosynthesis in breast tissue.

Studies have demonstrated that MCF-7 cells are sensitive to TNF- α -induced apoptosis and that TNF- α is an effective inducer of apoptosis in MCF-7 cells (Watabe et al. 2004; Kuo et al. 2005; Modiano and Bellgrau 2016). In this study, protein hydrolysate treatment induced the increases in expressions of TNF- α , Fas, and caspase-8 in MCF-7 and MDA-MB-231 cells. These data suggest that the protein hydrolysate stimulated apoptosis by inducing death receptor-mediated pathway. Previous studies have demonstrated the importance of Bax/Bcl-2 ratio in regulating the mitochondria-mediated apoptotic pathway in human breast cancer models (Shim et al. 2007). Our data showed that protein hydrolysate treatment increased the expression of Bax while down-regulating the Bcl-2 expressions, which happened along with the increased expressions of caspase-9 and apoptosis in MCF-7 and MDA-MB-231 cells. Therefore, these results clearly indicate that the mechanism of protein hydrolysate-induced apoptosis by mitochondrial-mediated pathway involves the mitochondrial membrane potential change *via* controlling the Bax/Bcl-2 ratio. Previous studies suggest that down-regulation of ErbB-2 could efficiently suppress the breast tumor cell growth (Chen et al. 2008, 2014). In this study, protein hydrolysate treatment showed a down-regulating effect on ErbB-2 expression in MCF-7 and MDA-MB-231 cells, and thus suggest that protein hydrolysate may enhance the apoptotic signal by suppressing the expression of ErbB-2.

In conclusion, potential therapeutic value of protein hydrolysate of *A. tarichi* roe and further research in animal tumor models is necessary to confirm its anti-cancer activity *in vitro*. The impact of this study provides information on cell specific pathways affected by this protein hydrolysate and could open avenues for the use of the protein hydrolysate of *A. tarichi* roe as a nutraceutical diet/drug for breast cancer

prevention. In addition, this research provides insight on the molecular mechanism of action of the protein hydrolysate of *A. tarichi* roe against human breast cancer cells and results on its potential drug-like property. The production of this protein hydrolysate is less expensive than general cancer drug and could be a promising alternative strategy to current expensive anti-cancer therapies.

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Conflict of interest. The authors do not have any conflict of interest to declare.

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