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Assessment of the Inhibitory Effects of Ficin-hydrolyzed Gelatin Derived from Squid (*Uroteuthis duvauceli*) on Breast Cancer Cell Lines and Animal Model

Sogol Shahidi[1], Shahla Jamili[1], Pargol Ghavam Mostafavi[1], Sassan Rezaie[2], and Mohammadreza Khorramizadeh[3]

[1] Department of Marine Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Division of Molecular Biology, Department of Medical Mycology and Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[3] Biosensor Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Marine novel natural products have been applied for cancer therapy. Enzyme-digested gelatin hydrolysates have proven to serve as promising sources of potent biologically active peptides.

Potential anti-breast cancer properties of the extracted Ficin-digesterd gelatin hydrolysate from Indian squid (*Uroteuthis duvauceli*) was extensively characterized by cellular and animal models. Gelatin was extracted from squid skin, hydrolyzed by Ficin, and characterized by standard physico-chemical methods. Ficin-digested gelatin hydrolysate was used at various doses of 0-0.1 mg/mL for assessment of MCF-7 and MDA-MB-231 breast cancer cells versus HUVEC normal cells. Cytotoxicity, phase-contrast morphological examination, apoptosis/necrosis, clonal-growth, cell-migration, Matrix-metalloproteinases (MMPs) zymography, and Western blotting were used for cellular assessments. For animal studies, breast tumor-induced BALB/c mice received hydrolyzed gelatin regimen, followed by tumor size/growth and immune-histochemical analyses.

Significant inhibition of MCF-7 and MDA-MB-231 with no cytotoxicity on HUVEC cells were detected. Apoptosis was increased in cancer cells, as revealed by elevated ratio of cleaved caspase-3 and PARP. MMP-2 and MMP-9 activities in both cancer cells were diminished. In mice, gelatin hydrolysate prevented weight loss, decreased tumor size, induced p53, and down-regulated Ki67 levels.

These findings suggest that Ficin-digested gelatin hydrolysate could be a beneficial candidate for novel breast cancer therapies.

Keywords: Apoptosis; Breast cancer; Ficin-hydrolyzed; Functional food; Matrix metalloproteinase squid gelatin

Corresponding Author: Mohammadreza Khorramizadeh, PhD; Biosensor Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran. PO.Box: 1411713137. Tel: (+98 21) 8863 1298, Fax: (+98 21) 8822 0052, E-mail: khoramza@tums.ac.ir

INTRODUCTION

The marine ecosystem is the richest reservoir of organisms containing digestible proteins and functional

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foods. Apart from providing fishery products, marine animals can generate a wide variety of medicines, nutraceuticals, novel enzymes, and bioactive and industrial compounds for food and healthcare. Recent scientific pursuit has thrown newer insights into the nutritional and therapeutic values of bioactive peptides components from these marine organisms.[1] In particular, aquatic products and by-products have been proven to be potential sources of protein based products and bioactive peptides.[2]

Gelatin is one of the valuable proteins that is derived from by-products and wastes, generated during animal slaughter and processing of marine sources by heat denaturation. This fibrous protein is widely used in food, pharmaceutical, cosmetic and photographic applications because of its unique chemical and physical characteristics.[3] Commercial gelatins are mostly obtained from bovine and porcine skins and bones. However, the use of gelatin from these resources is getting restricted, owing to the potential transmission of pathogenic vectors as well as some religious bans.[3,4] Consequently, marine gelatin has gained increasing interest as the potential alternative for other gelatin sources like land animal counterpart.[5] Characteristics and functional properties of different marine species skin and bone gelatin has been intensively studied.[5] Some fish and squid species skin gelatins have been reported to give rise to biologically active peptides with antioxidant, antimicrobial and antihypertensive activity, but a few references can be found in the scientific literature regarding anticancer effects of marine species gelatin.[5]

Cancer is one of the largest single cause of death in human, and breast cancer is the most common cancer among women worldwide.[6,7] The high morbidity and mortality are mainly attributed to metastasis, which generally contains invasion, intravasation, extravasation, circulation, and colonization steps in lung, bone, and liver.[8] Surgical resection, radiotherapy and chemotherapy as conventional cancer therapy methods have been extensively applied. Despite remarkable therapeutic advances, frequent resistance to anticancer drugs still pose main problems.[5] Therefore, recent research and development of more effective and less toxic natural health products have become essential by the pharmaceutical industry, with growing interest in the identification and characterization of natural antitumor agents. Measuring the cytotoxic properties of a natural health product compound against cancer cells provides beneficial intuitions into chemoprotective and/or chemotherapeutic potential agents as an effective treatment.[9]

Among marine species, squid is one of the essential fishery product in southern seas of Iran. Squid skin, a by-product with low market value, is rich in gelatin and has recently been used for extraction to increase profitability.[5]

The molecular weight distribution and amino acid composition of extracted gelatin are affected by squid processing conditions and are main factors shaping the functional and biological properties of gelatin.[10] Controlled enzymatic hydrolysis of protein compounds can produce a series of small polypeptides which can modify and even improve the protein functional and even biological characteristics.[11] Interestingly, gelatin-hydrolysate amino acid composition resulting from the hydrolysis with different type of enzymes reserves similarities with that of the parent gelatins.[12,13]

Regarding squid skin gelatin, to the best of our knowledge, there paucity of reports on possible anticancer activity of gelatin derived peptides or hydrolysates. However, there have been some investigations describing some cytotoxic and anti-proliferative effects on breast cancer cells for gelatin hydrolysates prepared by enzymatic digestion of giant squid skin gelatin with seven commercial proteases.[14] Inhibition of apoptosis as a physiologic process of programmed cell death has been widely recognized as a mark of most types of cancer. Therefore, focus on inducing apoptosis in cancer therapies by natural health products with fewer side effects on normal cells, has recently been pursued by researchers.[15]

In the present study, gelatin has been extracted from Indian squid skin and hydrolyzed with a novel natural and non-toxic protease, Ficin.

Ficin-hydrolyzed gelatin then assessed for potential cancer cell inhibition and pro-apoptosis effects by using in vivo and in vitro experimental models.

MATERIALS AND METHODS

Squid skin Gelatin Preparation

Indian squid (*Uroteuthis duvauceli*) were caught from the Persian Gulf and the squid outer tunics were removed and frozen at -20°C. Gelatin was extracted according to the method of Grossman and Bergman.[16] The supernatant viscous gelatinous mixture was dried at 42°C until moisture content was less than 15%

and stored at -20°C.

Enzymatic Hydrolysis of Gelatin

Squid skin gelatin was dissolved in water (2.5% W/V), then Ficin from fig tree latex, (Sigma-Aldrich, MO, USA) was added with an enzyme to substrate ratio of 1:30 (W/W). The reaction was conducted within 3 h, under optimal conditions (*p*H 7.5, 60°C)[17] in order to produce bioactive peptides. The pH was monitored and adjusted during the hydrolyzing process. The enzyme was inactivated by heating the mixture at 90°C for 10 min. Un-hydrolyzed aggregates were removed by centrifugation and the clear supernatant were collected, lyophilized and stored at -80°C. Furthermore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) used for analyzing the apparent molecular weight distribution of the hydrolyzed molecules using 5% stacking gel and 15% resolving gel, followed by Coomassie blue staining.[18,19] Bovine hide gelatin Type I (Sigma-Aldrich, MO, USA) were used as markers of α-chain, β-chain and γ-component motilities.

Determination of Amino Acid Composition

In order to determine the amino acid composition, 1 mg of the extracted gelatin and enzymatically digested gelatin were acid-hydrolyzed in 1 mL of 6 N HCl at 110°C for 24 h, then mixed with norvaline (Sigma-Aldrich, MO, USA). The resulting mixture were vacuum-dried, dissolved in application buffers and were injected into a RF-20A high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan), equipped with fluorescence detector and Knauer C18 reversed-phase column. Running time was carried out for 0-50 minutes until amino acids separation completed. All measurements were performed in triplicate.

Cell Culture and Cell Lines

A couple of human breast cancer adenocarcinoma cell lines including MCF-7 (estrogen positive breast cancer cells) and MDA-MB-231 (estrogen negative breast cancer cells), as well as 4T1 (mice breast cancer cell line) and primary human umbilical vein endothelial cell line (HUVEC) were purchased from the National Cell Bank of Iran (NCBI, Tehran, Iran). All cell lines were cultivated and maintained in optimal conditions in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin; at 37°C in a 5% CO2 and 95% air humidified incubator.[20]

Cell Growth Inhibition Assay

The possible anti-proliferative effects of squid skin gelatin hydrolysate on breast cancer cell lines as well as HUVEC cells were determined by MTT (3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma-Aldrich, MO, USA) assay according to the method of Kumar et al.[21] In brief, cells were seeded at a density of 1×10^4 cells /well in a final volume of 200 µL in 96-well plates and various concentrations (0.0005, 0.001, 0.005, 0.01, 0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate was added to the determined wells in triplicate manner. After 24 h incubation, 15 µL of 5 mg/mL MTT solution was added to each well. Following 3 h incubation, the medium of each well was removed and dimethyl sulfoxide (DMSO) was added to stop succinate-tetrazolium reductase activity and solubilize the insoluble formazan crystals. The spectrophotometric absorbance was determined by Microplate Reader (BioTek, USA) at 570 nm. The results were expressed as relative percentage of cell growth of each well to the control cells. IC_{50} values (50% inhibitory dose) were defined as the gelatin hydrolysate concentrations causing 50% inhibition of cell growth compared to the control.

Assessment of Morphological Changes in Cells

To study the cell morphological changes, MCF-7, MDA-MB-231 and HUVEC cells were seeded in 24 well plates at a density of 1×10^5 cells /well and grown for 24 h until 80% confluency reached. Different concentrations (0.01, 0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate were added to each well and cells were incubated for 24 h at 37°C, 5% CO_2 and 95% air. To determine morphological changes, the phase-contrast microscopy (Zeiss, Germany) was used at 200× magnification.

Detection of Apoptotic Versus necrotic Cells

Determination of apoptotic and necrotic cells was performed by using ethidium bromide/acridine orange (EB/AO) double staining assay. Cells were seeded at a density of 5×10^5 cells /well in culture plates and after treatment with different concentrations (0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate for 24 h, the cells stained with a mixture of AO (100 mg/mL) and EB

(100 mg/mL) (Sigma-Aldrich, MO, USA). The stained cells were scrutinized immediately by means of a fluorescence microscope (Zeiss, Germany) at 100× magnification. Untreated cells were observed as controls. In each experiment, more than 300 cells were counted. Results were expressed as Means±SEM for three independent determinations.[22]

Clonal Growth Assay

Clonal growth assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. Reduction in tumor cells clonal survival was assessed by the modified clonogenic assay protocol.[23] A colony is defined as a bunch of at least 50 cells. To evaluate the ability of MCF-7 and MDA-MB-231 cells colony formation, cells at density of 1×10^3 cells/plate on to the 50 mm culture plates were treated with different concentrations (0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate for 24 h in triplicate. Culture media were changed every 3 days until cells in control plates formed colonies with considerably good size (50 cells per colony is the minimum for scoring). Cells were then fixed by acetic acid/methanol ratio of 1:7 (V/V) for 20 min and stained with crystal violet. Number of colonies were counted with stereomicroscope at 100× magnification.

Cell Migration Assay

To assess cell motility and migration ability, a modified wound-healing model was used.[24] In brief, MCF-7 and MDA-MB-231 cells were seeded with 1×10^6 cells per well in 6-well plates and allowed to grow to approximately 95% confluence. The confluent cell monolayers were incubated in the absence (as the vehicle control) or presence of different concentrations (0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate for 24 h. A horizontal incision was made with a 10 µL plastic pipette tip at the center of each well. Three random views along the scraped line were photographed in each well at 100× magnification at 0 and 24 h with a light phase contrast microscope (Olympus Optical, Tokyo, Japan) to evaluate the inhibition capability of gelatin hydrolysate on cell migration. The image was analyzed and average scraped width of each well measured and compared with control.

Gelatin Zymography

Gelatin zymography technique was used to measure the activity of secreted matrix-metalloproteinases (MMP-2 and MMP-9) as previously described.[25] MCF-7 and MDA-MB-231 cells were seeded in a conditioned-medium, then treated with different concentrations (0.01, 0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate in RPMI 1640 assay medium containing 0.5% FBS. Untreated cells were used as control. Culture media were collected 24 h post treatment and centrifuged at 1500 rpm for 5 min at 4°C to remove debris. Samples were normalized based on their protein content, mixed with non-reducing sample buffer and separated by electrophoresis on 7% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gel was immersed in 2.5% Triton X-100 and subsequently incubated in enzyme buffer for 24 h at 37°C. Gel was stained with 0.5% Coomassie blue stain. The unstained bands correspond to the areas of proteolytic activity and gelatin digestion. Each experiment was carried out in triplicate.

Western Blotting Analysis

MCF-7 and MDA-MB-231 cells were treated with different concentrations (0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate. Cells collected and the protein concentration was calculated and adjusted by using Bradford's method.[26] Equivalent amounts of proteins were separated by SDS-PAGE electrophoresis and transferred onto a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was then blocked with 5% non-fat dry milk in Tris-Buffered-Saline with Tween (TBST) for 1 h at room temperature, and incubated with primary antibodies overnight at 4°C. This was followed by incubation with secondary antibody for 1 h at room temperature. After extensive washing with TBST, the Electro chemi-luminescence (ECL) reagent was used to measure the chemi-luminescence intensity. β-actin was used as a loading control in entire western blotting analysis. The result was analyzed by measuring integrated density with Image J software.[27]

Breast Cancer Animal Model Experiment

The entire animal experiment was performed in the framework of grant coded 15882, approved on January 10th, 2012, by the "Research Council" of Tehran University of Medical Sciences, Tehran, Iran. Ethical guidelines for the care and use of laboratory animals were in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH publications no. 80-23). Six-weeks-old inbred

female BALB/c mice, purchased from Pasteur Institute (Tehran, Iran), were housed in a barrier with 12 h light/dark cycles at 22–24°C in an utterly designed pathogen-free isolation facility with food and water ad libitum at all times. One week before experimentation, animals were allowed to adapt. 1×10^6 logarithmic growth phase 4T1 cells/200 µL were subcutaneously injected into the right flank of each treated mice. Ten days after inoculation, tumor mass was established. 21 mice were randomized into three groups (7 mice/group) for the following treatments. The mice were orally administrated with gelatin hydrolysate (1 mg/1 mL/kg) daily for 32 days. The body weight of the mice and the tumor size were recorded everyday throughout the treatment period. Tumor size was measured using a digital Caliper, and volume (mm^3) was calculated using the following formula: Length×width2×π/6. Mice were sacrificed one month after starting treatment. The developed tumors and the main organs, including the liver, kidney, and spleen were excised, weighed and fixed in 10% formalin for following analysis. Organ weights were expressed as a percentage of body weight.

Tissue Preparation for Histological Evaluation and Immune-Histochemical Staining

The resected tumor was fixed in 10% formalin, embedded in paraffin and then sectioned. Tumor sections (5 µm) were stained with hematoxylin and eosin (H&E) subsequently photographed using a camera coupled to microscope (Olympus-BX51BX51, America, Center Valley PA) at 400× magnification for analyzing morphological and pathological evaluation of the samples. Tumor sections were also stained by immunohistochemistry. To assess expression of p53 and Ki-67 (those are considered among the most sensitive markers of the proliferative cellular potential), an immune-histochemical staining (IHC), was used as previously described.[28] In the current study, sections after blocking endogenous peroxidase using 3% hydrogen peroxidase, treated with primary mouse anti-human monoclonal Ki67 and p53 antibodies were diluted 1:100 in phosphate-buffered saline containing 0.1% Tween-20 (PBST) and 5% bovine serum albumin and subsequently with the secondary avidin-biotinperoxidase and DAB (3,3'-diaminobenzidine tetrahydrochloride) antibodies. Sections were counterstained briefly with hematoxylin. Bright-field images of immune-stained tissues were viewed on microscope (Olympus-BX51BX51, America, and Center Valley PA) with digital camera for images acquisition.

Statistical Analysis

Statistical analyses were conducted using SPSS version 22 (IBM Corp., Armonk, N.Y., USA). All data were expressed as Mean±SEM. Statistical significances between the groups were determined by one-way analysis of variance (ANOVA) followed by a specific post hoc test to analyze the differences. A value of p less than 0.05 was considered to be statistically significant.

RESULTS

Molecular Weight Distribution of Gelatin and Gelatin Hydrolysate

The electrophoretic (SDS-PAGE) patterns of squid skin gelatin and gelatin hydrolysate are shown in Figure 1. Apart from other factors, functional properties of gelatin are influenced by the distribution of molecular weight and composition of its subunits. Extracted gelatin was characterized by bovine hide gelatin Type I profile, with a notable amount of α-chains (α_1/α_2 ratio around 2), a considerable presence of β-components (α-chain dimers) and particularly of higher molecular weight aggregates including γ–components (α–chain trimers).

Amino Acid Composition

The amino acid composition of the squid skin gelatin and gelatin hydrolysate, compared to the bovine hide gelatin, expressed as residues per 1000 total amino acid residues, are shown in Table 1. One of the most predominant amino acid in gelatin was glycine and showing approximately 1/3 of the total amino acids and it was the major component. The amino acid composition of squid skin gelatin hydrolysate is very similar to that of the parent gelatin; both being rich in residues of alanine, argenine, aspartic acid/asparagine, glutamic acid/glutamine, glycine, proline and hydroxyproline.

Ficin-Digested Gelatin Hydrolysate Significantly Inhibited Cell Growth

To investigate the effect of squid skin gelatin and gelatin hydrolysate on cells growth, a dose-response

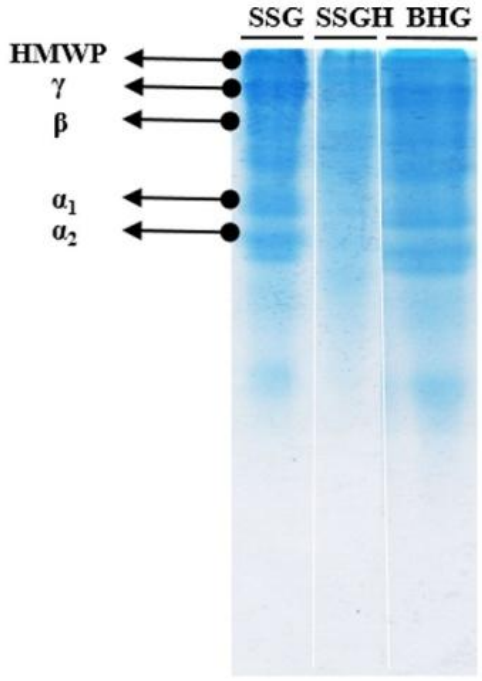

Figure 1. Considerable hydrolytic activity of ficin protease on squid skin gelatin. Electrophoretic patterns of squid skin gelatin (SSG) and squid skin gelatin hydrolysate (SSGH) were characterised by bovine hide gelatin (BHG) Type I profile as markers of α-chain, β-chain and γ-component motilities. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis used for analyzing the apparent molecular weight distribution, using 5% stacking gel and 15% resolving gel, followed by Coomassie blue staining. α–chains, β and γ -components are clearly predominant in the electrophoretic profile of squid skin gelatin whereas hydrolyzed gelatin consisted of more degraded protein material without clear peptide bands.High molecular weight protein (HMWP) on the top of the polyacrylamide gel in both gelatin and gelatin hydrolysate is shown.

experiment was performed on MCF-7, MDA-MB-231and HUVEC cells. The pH value of the gelatin and gelatin hydrolysate were 6.7 and 7, respectively, in cellular analysis. Squid skin gelatin did not show inhibitory effect on cell growth (data not presented).

However, Ficin-digested gelatin hydrolysate exerted a significant inhibitory effect on the growth of both MCF-7 and MDA-MB-231 in a dose-response manner with an IC_{50} of approximately 0.02 and 0.05 mg/mL, respectively. Conversely, HUVEC cells growth remained almost un-affected; only a significant growth-decrease was detected when treated with high (0.1 mg/mL) concentration of Ficin-digested gelatin hydrolysate (Figure 2).

Cell Morphological Changes Was Detected after Ficin-Digested Gelatin Hydrolysate Treatment

Morphological changes were examined under a phase-contrast microscopic exposure after MCF-7, MDA-MB-231 and HUVEC cells were treated with Ficin-digested gelatin hydrolysate. Figure 3 shows that gelatin hydrolysate induced morphological changes and cell membrane shrinkage. Apoptotic bodies were observed and cells were broken into smaller pieces and bubble-like blebs developed on the membrane in MCF-7 and MDA-MB-231 cells.

Ficin-Digested Gelatin Hydrolysate Induced A Significant Dose-dependent Increase of Apoptosis in Treated Cells

Double staining of two breast cancer cell lines are shown in Figure 4. In EB/AO fluorescent staining, cells were specified as viable, apoptotic and necrotic categories according to the fluorescence emission and the stained nuclei. Viable cells have green nuclei and apoptotic and necrotic cells appear to bear yellow and red nuclei, respectively. The maximum increase in the number of apoptotic cells were observed in 0.1 mg/mL concentration of gelatin hydrolysate in both cell lines. Necrotic cells appeared in a very small percentage.

Marked Clonal Repression Was Induced by Ficin-Digested Gelatin Hydrolysate

A clonal growth assay in MCF-7 and MDA-MB-231 breast cancer cell lines that are shown in Figure 5, confirmed that treatment with Ficin-digested gelatin hydrolysate, conferred a marked repression of clonal growth. In both cells, at the maximum experimental dose (0.1 mg/mL), gelatin hydrolysate strongly inhibited the clonal growth; it was about 87.6% and 91.4% in MCF-7 and MDA-MB-231 cell lines, respectively.

Ficin-Digested Gelatin Hydrolysate Inhibited Cell Migration of MCF-7 and MDA-MB-231 Cells

Motility response of MCF-7 and MDA-MB-231 cells to different concentrations of squid skin gelatin hydrolysate were shown in Figure 6. Treatments with Ficin-digested gelatin hydrolysate, renders the cracks unpopulated by migratory cells, whereas in the control

Table 1. Amino acid composition of Ficin-digested squid skin gelatin hydrolysate (SSGH) reserves similarities with squid skin gelatin (SSG) as a parent gelatin. Data were compared to the bovine hide gelatin (BHG) and expressed as residues per 1000 total amino acid residues. Data were expressed as Mean±SEM (N=3). Standard error of the mean were in all cases lower than 4%.

Amino acid	Number of residues/1000 residues		
	SSG	SSGH	BHG
Alanine	96.6	95.4	121.2
Arginine	53	52	43.2
Asparagine	0.2	0.1	0.7
Aspartic acid	74.4	74	55
Cysteine	1.1	1	0
Glutamic acid	99.4	97	87
Glutamine	1.7	1.2	1
Glycine	332.1	330.4	343.4
Histidine	5	6	4
Hydroxylysine	8	10	14
Hydroxyproline	70.1	66	48
Isoleucine	24.4	27	21
Leucine	28.5	31.2	26
Lysine	10	9	23
Methionine	3.4	3	1
Phenylalanine	14.2	14	15
Proline	79.9	85	121
Serine	40.2	43	25
Threonine	24.7	24	21.5
Tryptophan	0	0	0
Tyrosine	7.6	6.4	3
Valine	25.5	24.3	26
Total	1000	1000	1000

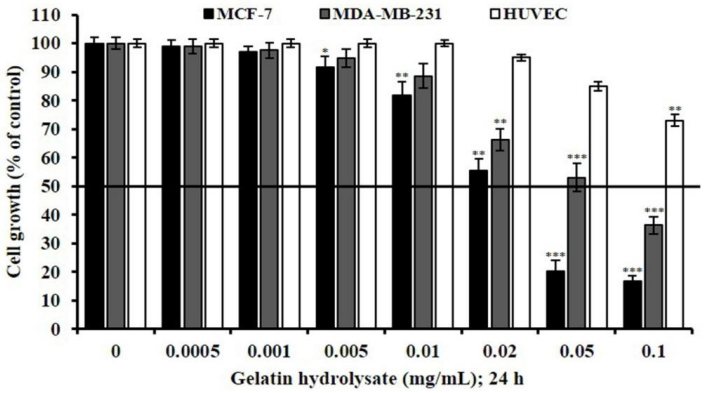

Figure 2. Ficin-digested gelatin hydrolysate significantly inhibits the growing of both MCF-7 and MDA-MB-231 cells in a dose-response manner. MCF-7, MDA-MB-231 and HUVEC cells were treated with different concentrations (0.0005, 0.001, 0.005, 0.01, 0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate for 24 h. Cell growth inhibition was assessed by MTT assay. HUVEC cells growth remained almost un-affected; only a significant growth-decrease was detected when treated with high (0.1 mg/mL) concentration of Ficin-digested gelatin hydrolysate. *Significantly different from control group cells. (*p< 0.05, **p<0.01 and ***p<0.001). Data were expressed as mean±SEM (N=3). (0 mg/mL no treated cells as a control group).

group, migratory cells healed the slit thoroughly, indicating the migration-inhibitory effect of gelatin hydrolysate on MCF-7 and MDA-MB-231 cells. At the most effective dose of (0.1 mg/mL), gelatin hydrolysate strongly inhibited the motility of both cells, it was about 95% and 93% in MCF-7 and MDA-MB-231 cell lines, respectively.

Matrix-Metalloproteinase Activity Was Significantly Hindered by Ficin-Digested Gelatin Hydrolysate in a Dose-Response Fashion

The activity of matrix-metalloproteinases, as assessed by zymography, was shown in Figure 7. The presence of MMPs activity resulted in clear bands, which was assigned to MMP-2 and MMP-9. At the most effective dose (0.1 mg/mL) of gelatin hydrolysate, densitometric analysis of the zymograms showed reductions of 87.6% and 83.4% MMP-2 and MMP-9 in MCF-7 cells and 67.3% and 63.7% MMP-2 and MMP-9 respectively in MDA-MB-231 cells, respectively, as compared to untreated control cells.

Cleavage of Apoptosis Markers, Caspase-3 and PARP, Was Markedly Induced by Ficin-Digested Gelatin Hydrolysate

Western Blot analysis was performed to determine caspase-3 expression and PARP cleavage in MCF-7 and MDA-MB-231 cells treated by squid skin gelatin hydrolysate (Figure 8). Results demonstrated that the gelatin hydrolysate significantly increased the level of caspsae-3 in treated cells, as compared to the untreated control cells. In parallel, a significant increase in cleaved PARP protein as a marker for cells undergoing apoptosis, in MCF-7 and MDA-MB-231 cells, was observed. This was confirmed by a significant PARP cleavage from 116 to 89 KDa observed following the treatment.

Ficin-Digested Gelatin Hydrolysate Decreased Tumor Growth In vivo

To investigate whether Ficin-digested gelatin hydrolysate treatment had any impact on tumor progression in vivo, female BALB/c mice were injected with murine breast cancer 4T1 cells and orally treated with gelatin hydrolysate for a period of 32 days. Body weight and the tumor size were recorded daily in tumor-bearing animals. Results indicate tumor size reduction in gelatin hydrolysate treated animals. Interestingly, mean tumor volume and weight in treated mice significantly decreased to 38.94% and 61.53%, respectively, as compare to the control group which were treated with PBS (Figure 9: A). Body and the main organs weight, including liver,

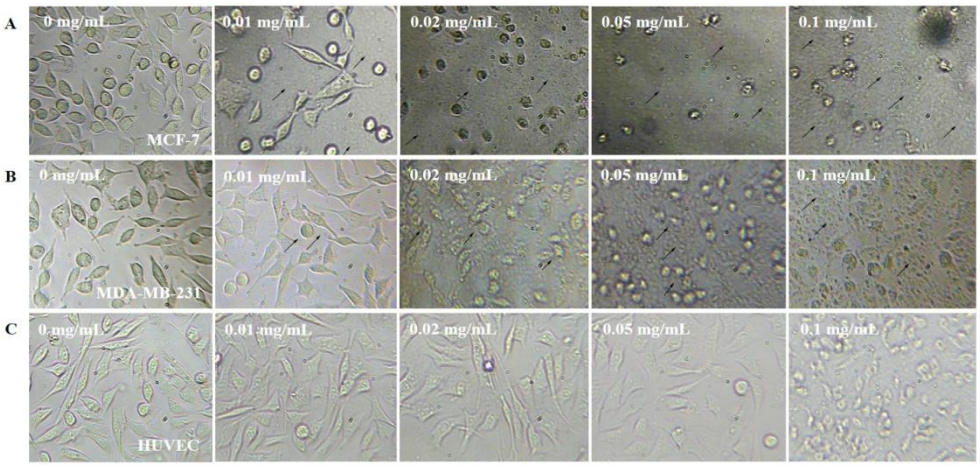

Figure 3. Ficin-digested gelatin hydrolysate induces morphological changes and cell membrane shrinkage in MCF-7 and MDA-MB-231 cells in a dose-response manner. Cells were treated with different concentrations (0.01, 0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate for 24 h. The morphological changes of (A) MCF-7, (B) MDA-MB-231 and (C) HUVEC cells were examined under a phase-contrast microscope at 200× magnification. (0 mg/mL no treated cells as a control group).

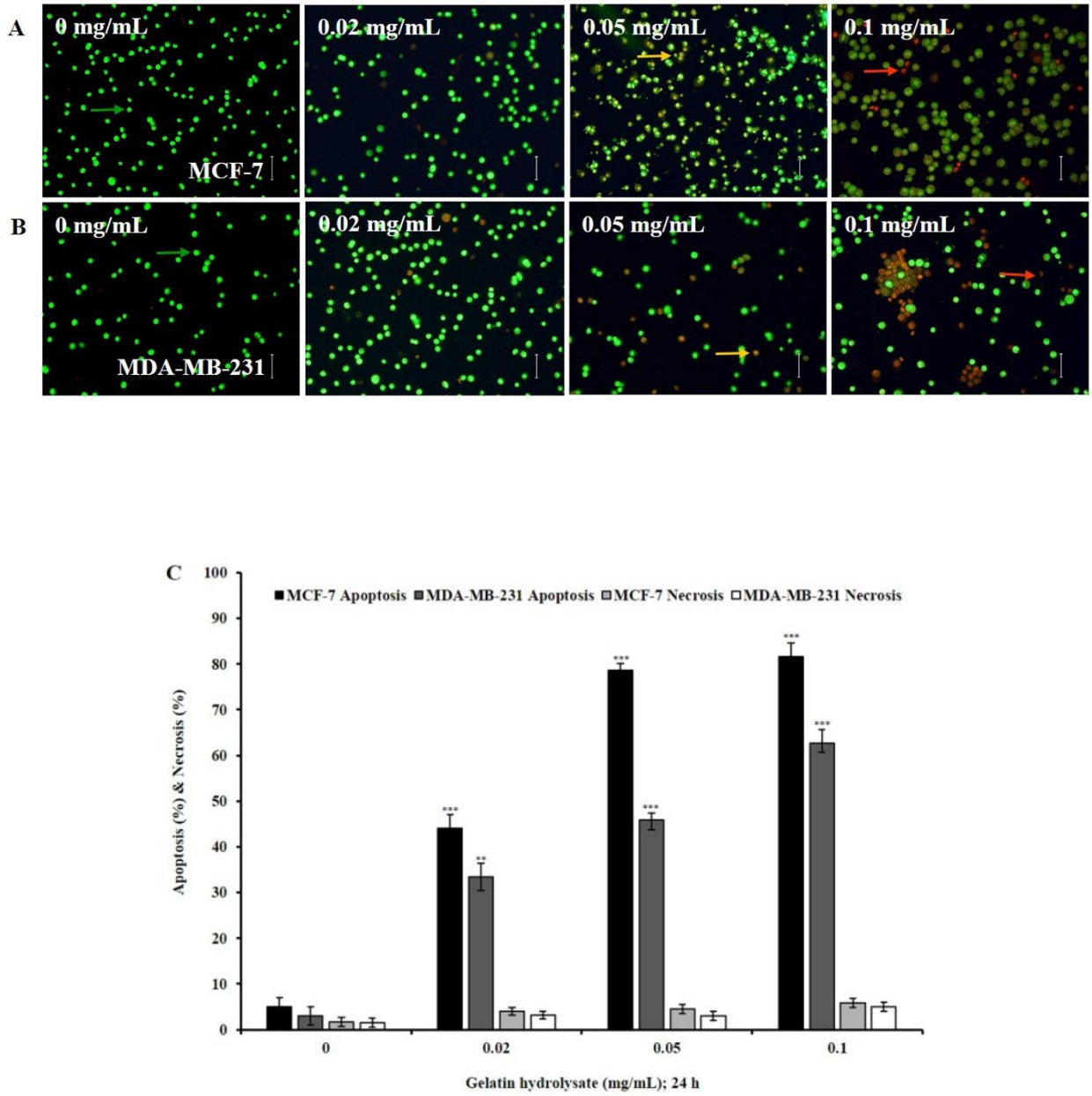

Figure 4. Ficin-digested gelatin hydrolysate increases apoptosis in MCF-7 and MDA-MB-231 cells. (A) MCF-7 and (B) MDA-MB-231 cells were treated with different concentrations (0.02, 0.05 and 0.1 mg/mL) of gelatin hydrolysate for 24 h and stained with a mixture of ethidium bromide/acridine orange fluorescent staining and scrutinized by means of a fluorescence microscope (magnification at 100×). Green, yellow and red cells (shown with green, yellow and red arrows) point to live, apoptotic and necrotic cells, respectively. Different values of (C) apoptotic and necrotic cells as percentage of all cells, measured by ethidium bromide/acridine orange fluorescent staining. Necrotic cells appeared in a very small percentage in both cell lines. *Significantly different compared to control group cells. (p<0.01 and ***p<0.001). Data were expressed as mean±SEM (N=3). (0 mg/mL no treated cells as a control group).**

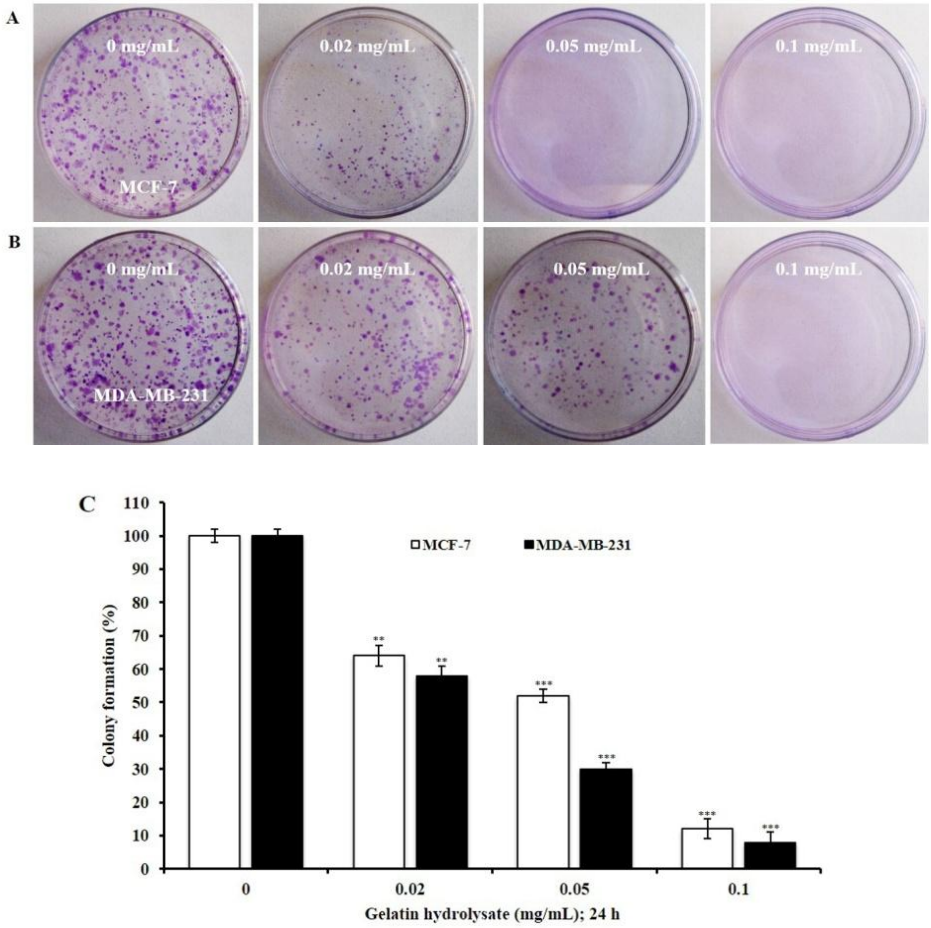

Figure 5: Ficin-digested gelatin hydrolysate represses clonal formation of MCF-7 and MDA-MB-231 cells. About 1×10^3 MCF-7 and MDA-MB-231 cells were seeded on to the 50 mm plates and after treatment with different concentrations (0.02, 0.05 and 0.1 mg/mL) of gelatin hydrolysate for 24 h, medium changed every 3 days. At the end visible colonies were fixed and stained. Morphological analysis of (A) MCF-7 and (B) MDA-MB-231 cells and (C) quantities analysis of inhibitory effect of gelatin hydrolysate are shown. Number of colonies were counted with stereomicroscope at 100× magnification. Data were expressed as Mean±SEM (N=3). *Significantly different from control group cells. (p<0.01 and ***p<0.001). (0 mg/mL no treated cells as a control group).**

kidney, and spleen in tumor-bearing mice are depicted in Figure 9: B. Results showed no reduction in body weight and main organs weight. Organs shape and size remained comparably normal in treated versus untreated animals. In addition, no death observed in any of the treated groups during the course of experiment.

Ficin-Digested Gelatin Hydrolysate Decreased the Nucleus to Cytoplasm Ratio, Decreased Ki67 and Increased p53 Protein Levels In vivo

Histological evaluation of breast cancer tumor-bearing animals, treated with Ficin-digested gelatin hydrolysate, is depicted in Figure 10: A and B. H&E staining of the resected tumor sections was used to determine the nucleus to cytoplasm ratio of tumor cells. Results showed that untreated tumors tissue, excised from control untreated tumor-bearing mice, consist of pleomorphic tumor cells with ovoid or round shape, high density and disordered cells. These tumors showed wide range of viable tumor cells. Nucleus to cytoplasm ratio of untreated cells were high. Conversely, gelatin hydrolysate-treated tumors showed

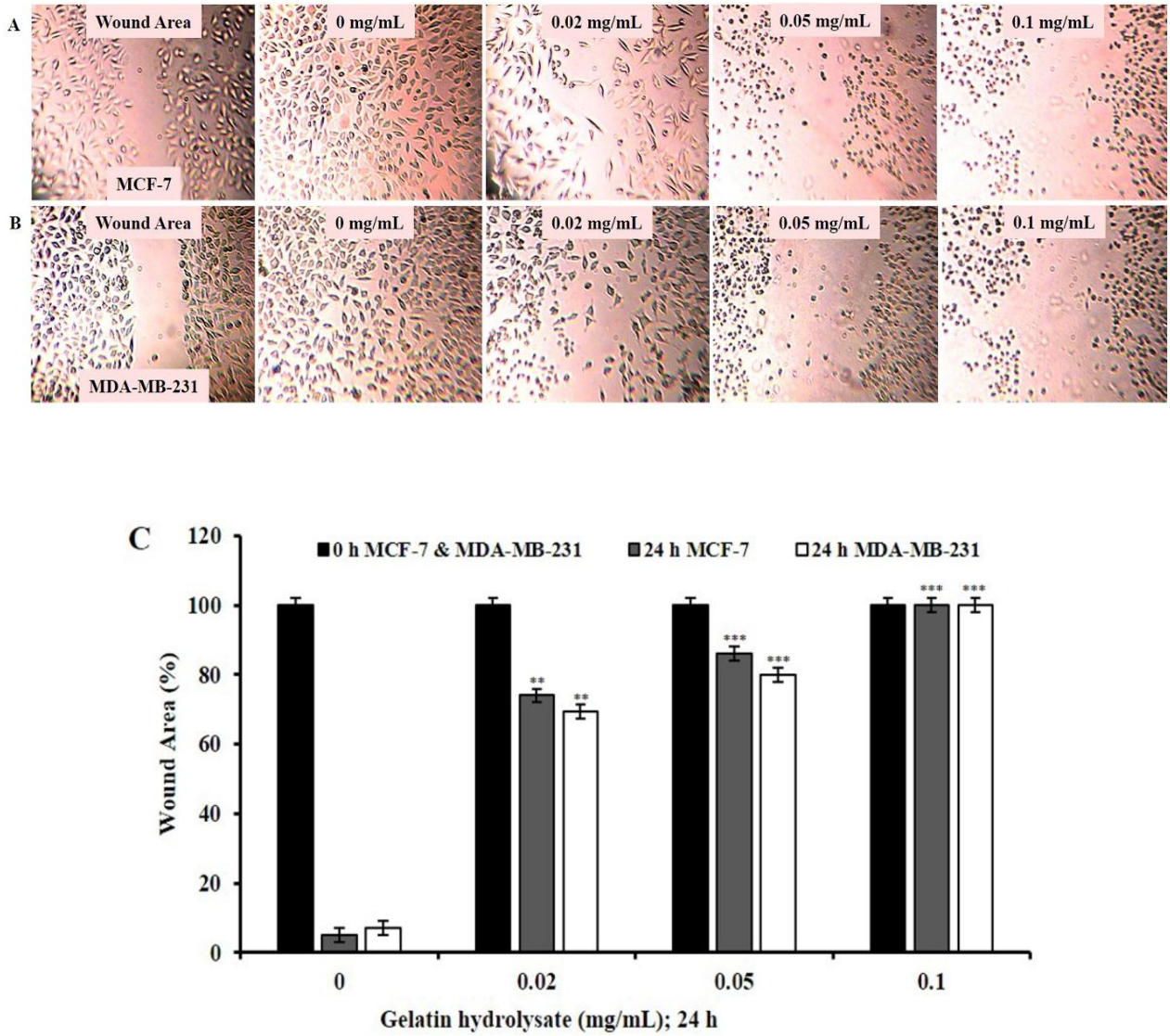

Figure 6: Ficin-digested gelatin hydrolysate inhibits the motility of MCF-7 and MDA-MB-231 cells. Cell motility inhibition was analyzed by a modified wound healing model. cells were seeded and treated with different concentrations (0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate for 24 h.Photographs were taken 0 and 24 h after wound edges were generated. Wound areas were evaluated with a light phase contrast microscope at 100× magnification. Morphological analysis of (A) MCF-7 and (B) MDA-MB-231 cells and (C) Quantitative analysis of the inhibitory effects of gelatin hydrolysate on cell motility after treatments are shown. Cell migration was analyzed by Image J software. Data were expressed as Mean±SEM (N=3). *Significantly different from control cells. (p<0.01 and ***p<0.001). (0 mg/mL no treated cells as a control group).**

a smaller amount of viable tumor cells and reduction of nucleus to cytoplasm ratio. In treated tumor tissues, polygonal nuclei were observed in majority of cells.

To further investigate whether the gelatin hydrolysate had any impact on tumor progression in vivo, immunohistochemistry analysis was used to assess p53 and Ki67 protein levels. As shown in Figure 10: C and D, Ki67 expression was strongly decreased in tumor tissues of the treated group. The expression of p53, however, was increased in treated group, as compared to control untreated tumor tissues.

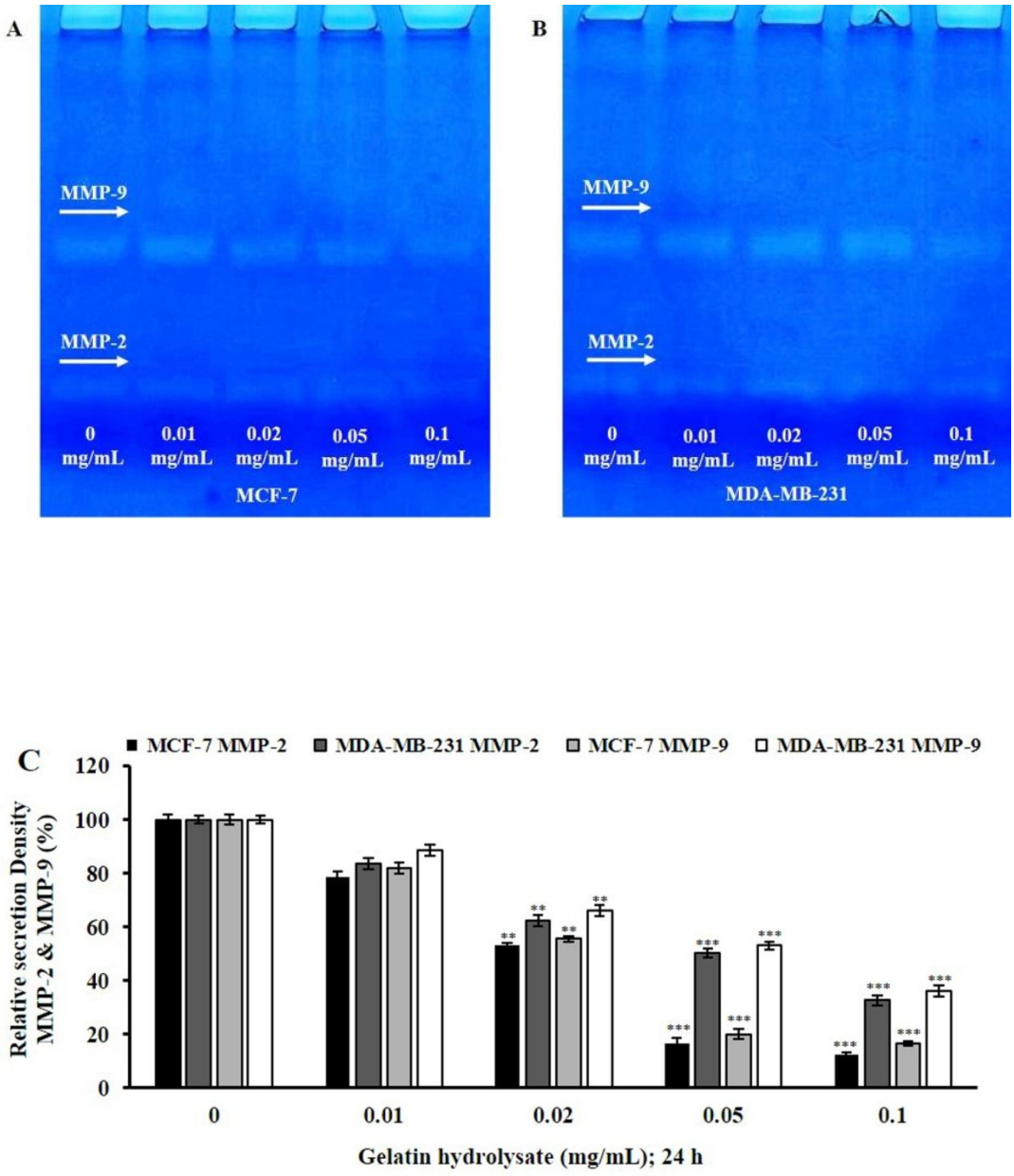

Figure 7. Ficin-digested gelatin hydrolysate reduces proteolytic activity of matrix-metalloproteinases (MMP-2 and MMP-9) in MCF-7 and MDA-MB-231 cells. Gelatin zymography technique was carry out. Culture media of treated cells with different concentrations (0.01, 0.02, 0.05, 0.1 mg/mL) of gelatin hydrolysate were separated by electrophoresis on 7% polyacrylamide gel containing 0.1% gelatin. Morphological analysis of proteinase (MMPs) presence in (A) MCF-7 and (B) MDA-MB-231 cells where resulted in clear bands and (C) quantitative analysis of the relative secretion density of MMP-2 and MMP-9 in MCF-7 and MDA-MB-231 cells are shown. Data were expressed as mean±SEM (N=3). *Significantly different from control cells. (p<0.01 and ***p<0.001). (0 mg/mL no treated cells as a control group).**

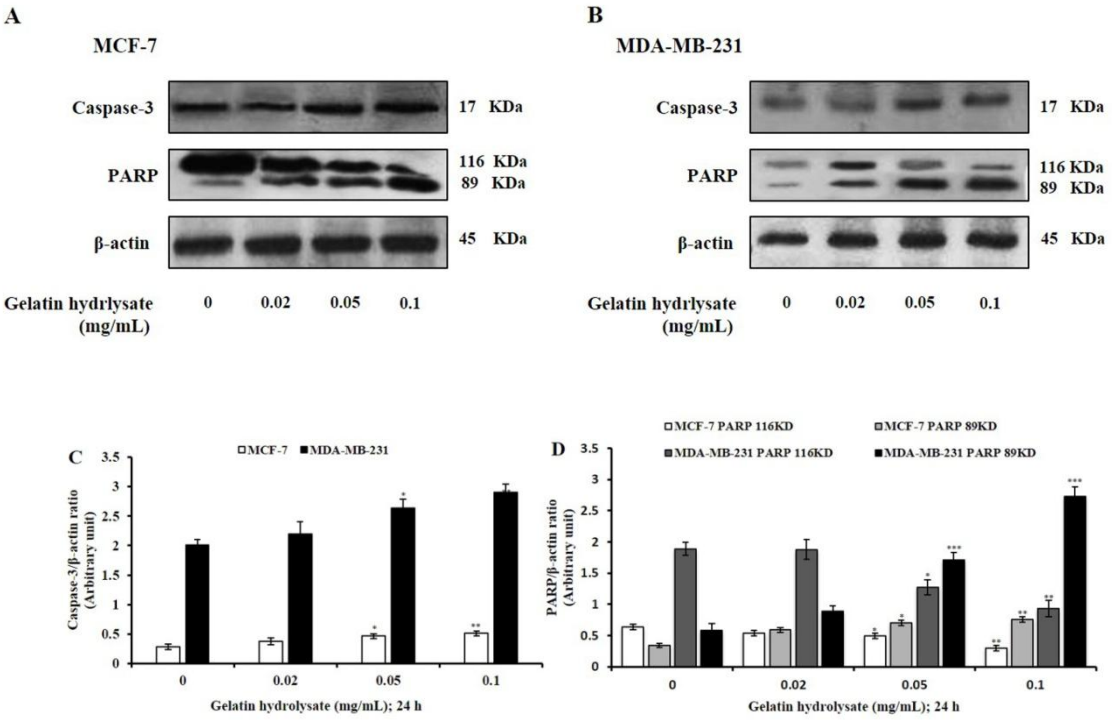

Figure 8. Ficin-digested gelatin hydrolysate increases the level of caspsae-3 and cleaved PARP proteins as markers for cells undergoing apoptosis, in MCF-7 and MDA-MB-231 cells. Cells were treated with different concentrations (0.02, 0.05 and 0.1 mg/mL) of gelatin hydrolysate for 24 h. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, western blotted, probed with anti-caspase-3 and anti-cleaved PARP antibodies and reprobed with anti-β-actin antibody. Caspase-3 and cleaved PARP levels in (A) MCF-7 and (B) MDA-MB-231 cells treated with gelatin hydrolysate are shown and the densities of (C) caspase-3 bands and (D) cleaved PARP bands in MCF-7 and MDA-MB-231 cells were measured and their ratio to β-actin were calculated. Data were expressed as mean±SEM (N=3). *Significantly different from control cells. (*p< 0.05 and **p<0.01). (0 mg/mL no treated cells as a control group).

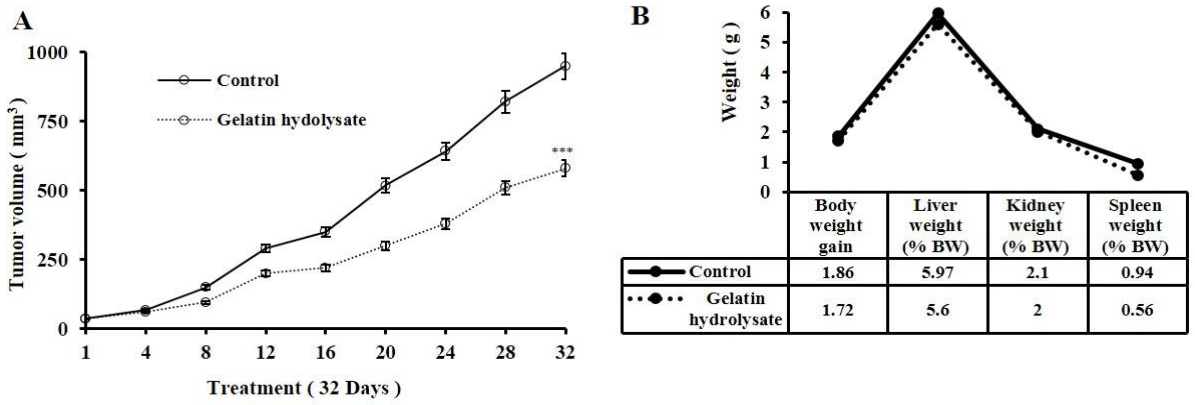

Figure 9. Ficin-digested gelatin hydrolysate decreases tumor growth in vivo with no side effects on vital organs. Female BALB/c mice were injected subcutaneously with 4T1 cells and after tumor mass establishment, orally administrated with gelatin hydrolysate (1mg/1mL/kg/day) for 32 days. (A) Tumor volume and (B) body and main organ (liver, kidney and spleen) weight gain were measured following treatment of the 21 breast cancer tumor bearing mice compared to the first day. Each value represents as Mean±SEM from 7 mice per group. (*p<0.001 as compared with control). (No treated mice as control groups).**

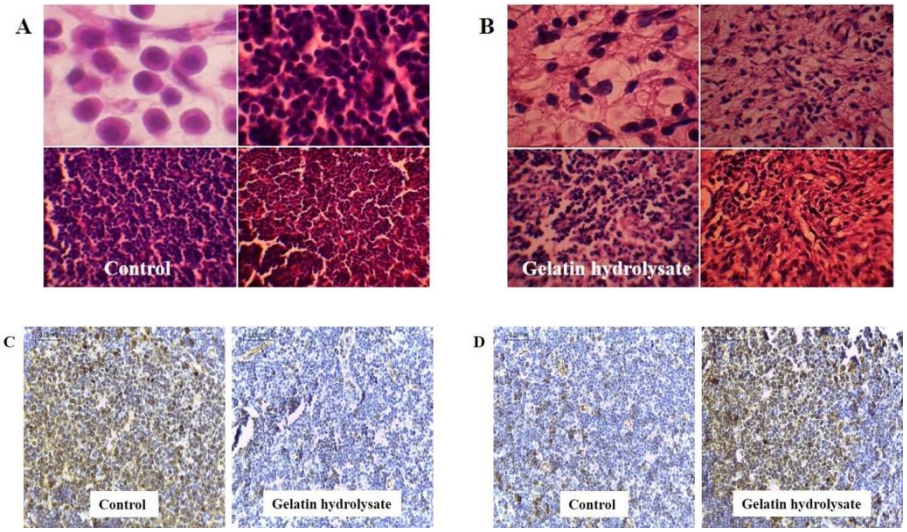

Figure 10. Ficin-digested gelatin hydrolysate decreases Ki67 and increases p53 protein levels and decreases the nucleus to cytoplasm ratio in vivo. Histology analysis was assessed by immune-histochemical and hematoxylin and eosin staining. Tumors resected from mice of the experiment were processed for tumor sections. Hematoxylin and eosin staining of (A) control mice compared to (B) treated mice showing smaller amount of viable cells and nucleus to cytoplasm ratio in treated group. Immune-histochemical staining of breast tumors in treated mice compared to control showing (C) Ki67 expression was strongly decreased and (D) p53 expression was significantly increased, indicated by brown nuclei. All magnifications ×50.

DISCUSSION

The notable aspect of chemopreventive properties of marine organisms as natural products and functional foods are mostly pertaining to the negligibly toxic or non-toxic feature of their consumption. Since chemotherapy side effects and drug resistance are major and challenging hindrance to cancer treatment, studies developed recently to cope with these problems. From another perspective, innumerable tones of byproducts from aquatic products processing which are considered as wastes, that most of them are discarded without any attempt at recovery, are potential sources of gelatin as a protein-rich product, with antioxidant and anticancer properties.[29,30] In a chemoprevention study, using lower concentration of an anticancer compound to reach the best cancer preventive effect, would be the ideal result. The doses of gelatin hydrolysate in this study are extremely low.

In this study, gelatin extracted from Indian squid skin was extensively analyzed by standard qualitative and quantitative techniques. Proximate composition determination, molecular weight distribution, unique enzymatic hydrolysis with commercial Ficin enzyme (sulfhydryl protease and family of cysteine proteases[31,32] that acts on bonds involving uncharged and aromatic amino acids)[33] were perforemed. In addition, amino acid composition test confirmed the presence of key amino acids components of squid skin gelatin as a protein compound. This allows that the growth inhibitory effect of this natural health product extract could be at least partly due to protein content. Evidently, molecular weight distribution of extracted squid skin gelatin, showed α-chains (α_1/α_2) as the major constituents. α–chains, β and γ -components are clearly predominant in the electrophoretic profile of squid skin gelatin whereas hydrolyzed gelatin consisted of more degraded protein material without clear peptide bands. Ficin-digested gelatin may be composed of a wide range of poly disperse products with varying degrees of hydrolysis. An appreciable amount of protein with high molecular weight aggregates on the top of the polyacrylamide gel was present in both gelatin and gelatin hydrolysate. This may be either due to the extraction temperature or, there is some aggregation and heat-stable cross-links formed during hydrolysis process. Ficin showed a

considerable hydrolytic activity on squid skin gelatin, as judged by SDS-PAGE results.

Amino-acid compositon analysis revealed that one of the most predominant amino acid in gelatin was glycine and showing approximately 1/3 of the total amino acids and it was the major component. Proline residues in squid skin gelatin hydrolysate was noticeably higher in comparison with the non-hydrolysate sample. This reveals a much higher degree of proline hydroxylation in squid hydrolysate. This a result of higher collagen cross linking in the squid gelatin, which was in fact the product of Ficin hydroxylation. High contents of the glycine, proline and hydroxyproline were obtained in both gelatin and gelatin hydrolysate. The hydroxylproline content was lower in hydrolysate. This result suggests a tendency of hydroxylproline to remain in the molecularly larger peptides. The presence of hydroxyproline is associated with small residual fragments in triple helix that hinder Ficin activity. Overall, a very similar amino acid composition was observed in both gelatin and gelatin hydrolysate (p>0.05). According to the results of the SDS-PAGE pattern and the amino acid composition of the squid skin gelatin and gelatin hydrolysate, Ficin showed an efficient hydrolysis on squid skin gelatin.

This study illustrates that gelatin hydrolysate at concentration of 0.1 mg/mL, 10-fold diluted than previous studies,[14] effectively inhibited the growth of MCF-7 and MDA-MB-231 that are two human breast cancer cell lines. In accordance with MTT assay, this finding suggests that gelatin hydrolysate showed a dose-dependent cytotoxic effect on MCF-7 and MDA-MB-231 cells. Ficin-digested gelatin hydrolysate elicited no morphological changes in HUVEC cells, except at the highest (0.1 mg/mL) dose.

Reports on esperase- or alcalase-digested gelatin hydrolysate showed cytotoxic effects on cancer cells.[14] Strikingly, our data showed that Ficin-digested squid skin gelatin hydrolysate decreased breast cancer cells growth by cytotoxic and antiproliferative effects, even more intensely than other proteases hydrolysates reported so far. Inhibition of apoptosis, is an obvious hallmark of cancer cells. In the present study, apoptosis was observed in the breast cancer cells treated with Ficin-digested gelatin hydrolysate as illustrated by the morphological assessment, apoptotic and necrotic staining. Compared with spontaneous apoptosis observed in control cells, a dose dependent increase in induction of apoptosis was observed in cells treated with Ficin-digested gelatin hydrolysate. Western Blot analysis was performed to determine the possible molecular mechanisms of apoptosis induced by squid skin gelatin hydrolysate. Cleavage of PARP and caspase-3 expression provides one of the most noticeable patterns in apoptosis.[34] Harmoniously, the employed histochemical and molecular methods of apoptosis in this study provided similar results, suggesting that Ficin-digested gelatin hydrolysate induces apoptosis in breast cancer cells in a dose-dependent fashion, and is directly correlated with the inhibition of cell growth. The induced apoptosis by Ficin-digested gelatin hydrolysate was evidenced, as well, by nuclear condensation, cell membrane shrinkage, and increased cleaved caspase-3 and PARP molecules in both MCF-7 and MDA-MB-231 cells, suggesting the triggered activation of apoptotic signaling pathways. In parallel, reduction in the colonogenic survival of tumor cells advocates that Ficin-digested gelatin hydrolysate can potentiate cancer cell killing. Production and activation of matrix-metalloproteinases break down the stroma and hence facilitate cancer cell metastasis.[35] There exist numerous report indicating over expression and secretion of MMP-2 and MMP-9 in highly metastatic tumors, induced in reaction to the stimulation occurred by different protein factors.[36] Treatment with Ficin-digested gelatin hydrolysate significantly reduced the gelatinolytic activities of secreted matrix-metalloproteinases (MMP-2 and MMP-9) in a dose dependent manner. Our results advocate that the triggered inhibition of cell migration by Ficin-digested gelatin hydrolysate could potentially be related to the repression of matrix-metalloproteinases (MMP-2 and MMP-9) activities.

In tumor-bearing animal experiments, Ficin-digested gelatin hydrolysate, administrated in BALB/c mice, inhibited tumor growth, as assessed by tumor size evaluation and p53 and Ki67 immunohistochemistry analyses. Results of Ki67 immunostaining, nuclear marker protein, correlated well with that of cellular proliferation and cell growth fraction of neoplastic cell populations, indicating that diminished tumor cell proliferation was closely aligned with the reduction of Ki67 expression in tumor-bearing mice treated with Ficin-digested gelatin hydrolysate. These accorded reductions were accompanied by increased expression of p53 protein, characterizing potential pro-apoptotic properties of Ficin-digested gelatin hydrolysate.

No marked toxicity or abnormal side effect was observed in vital organs of tumor-bearing mice treated with gelatin hydrolysate.

Data on in vitro assessments allowed us to demonstrate that Ficin-digested gelatin hydrolysate inhibited the proliferation and growth of breast cancer cells in a dose dependent manner and enhanced the apoptosis of MCF-7 and MDA-MB-231 cells. In vivo results, by generation of tumor mouse model of breast cancer suggest that gelatin hydrolysate inhibit the tumor growth, possibly by triggering apoptosis signaling. Our study indicated that Ficin-digested gelatin hydrolysate could be a potential natural health product in treatment of human breast cancer, though calling for further clinical trial investigations. In conclusion, the novel introduced Ficin-digested gelatin hydrolysate of Indian squid skin gelatin from Persian Gulf, exerted a potent cytotoxic and antiproliferative effects on the two breast cancer cell lines with respect to apoptosis activity in vitro and reduced the tumor growth of breast cancer in vivo. Collectively, this unique functional food hydrolysate of squid skin gelatin presented anticancer properties. The production of enzymatic hydrolysate with biological activities is an interesting alternative to the use of raw waste materials derived from the fishing industries. These data provide compelling evidence and propose that squid skin gelatin hydrolysates produced with Ficin digestion, could be employed in anticancer materials in combined natural health products and functional foods and supplements. Further confirmatory in vivo investigations are suggested and study in depth are needed in order to elucidate the nature and structure of the fractions of gelatin hydrolysates and discern the mechanisms of action.

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REFERENCES

1. Rao GS. Current status and prospects of fishery resources of the Indian continental shelf. 2010.
2. Ahmad M, Benjakul S. Characteristics of gelatin from the skin of unicorn leatherjacket (Aluterus monoceros) as influenced by acid pretreatment and extraction time. Food Hydrocoll 2011; 25(3):381-8.
3. Karim AA, Bhat R. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food hydrocoll 2009; 23(3):563-76.
4. Wilesmith J, Ryan J, Atkinson M. Bovine spongiform encephalopathy: epidemiological studies on the origin. Vet Rec 1991; 128(9):199-203.
5. Gómez-Guillén MC, Turnay J, Fernández-Dıaz MD, Ulmo N, Lizarbe MA, Montero P. Structural and physical properties of gelatin extracted from different marine species: a comparative study. Food Hydrocoll 2002; 16(1):25-34.
6. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Exp cell Res 2000; 256(1):42-9.
7. DeSantis C, Ma J, Bryan L, Jemal A. Breast cancer statistics, 2013. CA Cancer J clin 2014; 64(1):52-62.
8. Recht A, Solin LJ. Breast-conserving surgery and radiotherapy in early-stage breast cancer: the importance of local control. Semin Radiat Oncol 2011: Elsevier.
9. Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, et al. Inhibition of apoptosis during development of colorectal cancer. Cancer Res 1995; 55(9):1811-6.
10. Gómez-Guillén MC, Pérez-Mateos M, Gómez-Estaca J, López-Caballero E, Giménez B, Montero P. Fish gelatin: a renewable material for developing active biodegradable films. Trends Food Sci Technol 2009; 20(1):3-16.
11. Haard NF. Enzymic modification of proteins in food systems. Chem Func Prop Food proteins 2001; 155-90.
12. Kim SK, Kim YT, Byun HG, Nam KS, Joo DS, Shahidi F. Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. J Agric Food Chem 2001; 49(4):1984-9.
13. Gómez-Guillén M, López-Caballero M, Alemán A, López de Lacey A, Giménez B, Montero P. Antioxidant and antimicrobial peptide fractions from squid and tuna skin gelatin. Sea by-products as a real material: New ways of application 2010; 89-115.
14. Alemán A, Pérez-Santín E, Bordenave-Juchereau S, Arnaudin I, Gómez-Guillén M, Montero P. Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity. Food Res Int 2011; 44(4):1044-51.

15. Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. Cancer Lett 2008; 269(2):199-225.
16. Grossman S, Bergman M. Process for the production of gelatin from fish skins. Google Patents; 1992.
17. Barrett AJ, Kembhavi A, Brown M, Kirschke H, Knight C, Tamai M, et al. L-trans-Epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. Biochem J 1982; 201(1):189-98.
18. Bollag DM, Rozycki MD, Edelstein SJ. Protein methods: Wiley-Liss New York; 1996.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227(5259):680-5.
20. Liu L, Sun L, Wu Q, Guo W, Li L, Chen Y, et al. Curcumin loaded polymeric micelles inhibit breast tumor growth and spontaneous pulmonary metastasis. Int J Pharm 2013; 443(1):175-82.
21. Kumar D, Basu S, Parija L, Rout D, Manna S, Dandapat J, et al. Curcumin and Ellagic acid synergistically induce ROS generation, DNA damage, p53 accumulation and apoptosis in HeLa cervical carcinoma cells. Biomed Pharmacother 2016; 81:31-7.
22. Ogle AE. Ethidium Bromide/Acridine Orange Viability Staining Method. 2010.
23. Franken N, Rodermond H, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nature Protoc 2006; 1: 2315-9; PMID: 17406473. 2006.
24. Bimonte S, Barbieri A, Palma G, Luciano A, Rea D, Arra C. Curcumin inhibits tumor growth and angiogenesis in an orthotopic mouse model of human pancreatic cancer. BioMed Res int 2013; 2013:810423.
25. Khorramizadeh MR, Hosseinzadeh S, Safavifar F, Saadat F, Aalizadeh N, Falak R, et al. Interaction of CpG-oligodeoxynucleotides with Toll like receptor 9 induces apoptosis and modulates metaloproteinase-2 activity in human intestinal epithelium. Iran J Allergy Asthma Immunol 2007; 6(3):107-14.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72(1-2):248-54.
27. Mokhtarian K, Akhlaghi L, Meamar AR, Razmjou E, Naeini KM, Gholami S, et al. Serodiagnosis of fasciolosis by fast protein liquid chromatography-fractionated excretory/secretory antigens. Parasitol Res 2016; 115(8):2957-65.
28. Liu YX, Wang KR, Xing H, Zhai XJ, Wang LP, Wang W. Attempt towards a novel classification of triple-negative breast cancer using immunohistochemical markers. Oncol Lett 2016; 12(2):1240-56.
29. Mondal S, Bandyopadhyay S, K Ghosh M, Mukhopadhyay S, Roy S, Mandal C. Natural products: promising resources for cancer drug discovery. AntiCancer Agents Med Chem 2012; 12(1):49-75.
30. Benjakul S, Morrissey MT. Protein hydrolysates from Pacific whiting solid wastes. J Agric Food Chem 1997; 45(9):3423-30.
31. Mariant M, Camagna M, Tarditi L, Seccamani E. A new enzymatic method to obtain high-yield F (ab) 2 suitable for clinical use from mouse IgGl. Mol Immunol 1991; 28(1-2):69-77.
32. Milenic DE, Esteban JM, Colcher D. Comparison of methods for the generation of immunoreactive fragments of a monoclonal antibody (B72. 3) reactive with human carcinomas. J Immunol methods 1989; 120(1):71-83.
33. Creighton T. Disulphide bonds between cysteine residues. Protein Structure—a Practical Approach 1989:155-67.
34. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier G, Earnshaw W. Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 1994; 371(6495):346-7.
35. Siefert SA, Sarkar R. Matrix metalloproteinases in vascular physiology and disease. Vascular 2012; 20(4):210-6.
36. Rodríguez D, Morrison CJ, Overall CM. Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta 2010; 1803(1):39-54.