SAS Journal of Medicine
Abbreviated Key Title: SAS J Med
ISSN 2454-5112
Journal homepage: https://saspublishers.com

OPEN ACCESS

Biochemistry

Cytotoxic Activity of Whey Protein on Breast Cancer Cell in Vitro

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DOI: 10.36347/sasjm.2022.v08i08.006 | **Received:** 11.07.2022 | **Accepted:** 16.08.2022 | **Published:** 17.08.2022

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Abstract **Original Research Article**

Objectives*:** Natural products, which do not have any toxic effects in terms of human health, have an important place in cancer research today. In this study, we aimed to determine the anticancer effect of whey protein, which has no side effects, on breast cancer cell line in vitro. ***Method*:** Whey protein (whey) from cow's milk was obtained using the isoelectric point. The whey protein was sterilized by a membrane filter. Cell viability and IC50 values were determined by measuring the antitumor activity levels of different concentrations of lyophilized whey protein with the breast cancer cell line (Mcf-7) MTT viability test. In addition, cytokine levels (IL-2, IL-6 and TNF-α) of whey protein were measured. ***Results: Serial dilutions of whey protein starting from 6400 µg/ml and continuing with decreasing concentrations were incubated with Mcf-7 cells for 24, 48 and 72 hours. When incubated with cow whey Mcf-7 cells, cell viability at 24 hours was 45.57%; IC50 value 5.605 µg/ml, R2 = 0.968; Cell viability at 48 hours was 43.52%; IC50 value 5.257 µg/ml, R2 = 0.976; cell viability found in cow whey at 72 hours was 37.06%; IC50 value was determined as 3.144 µg/ml and R2= 0.373. ***Conclusion*:** It was concluded that whey protein has antiproliferative and anticarcinogenic effects in MCF-7 cancer cell lines**.** This suggests that whey proteins have the capacity to stimulate the production of cytokines and may support cytokine potentiation.

Keywords: MCF-7, cytotoxicity, whey protein, cytokine.

1. INTRODUCTION

Cancer refers to malignant tumors and it occurs as a result of uncontrollable growth and rapid division of the cells in the body. Constituting the fundamental structure of living beings, cells grow, divide, are aged and die, all within a certain cycle. Breast cancer is the type of tumor that causes uncontrollable growth and proliferation of the cells taking part in formation of milk and the milk ducts in breast tissue. Breast cancer is the most common cancer type in women that leads to death. Breast cancer contains such structures as human epidermal growth receptor-1, luminal A and luminal B which are found in estrogen and progesterone receptor [1].

Today, natural products which do not have any toxic effects on human health are considered to be important for cancer research. A certain amount of anticancer drugs is produced by synthesis of certain molecules in the nature [2]. Use of chemotherapy in treatment of patients with cancer is not only difficult and hard for some patients, giving rise to side effects, but it is also very much costly for country economies. Therefore, it is important that natural anticancer components be found that have very few side effects and of a low cost [3]. In recent years, there are studies where animal and plant-based natural components are used as a significant part of treatment of many diseases such as inflammation, infection and cancer [4, 5].

Breast Cancer Cell Line (MCF-7), is an invasive ductal carsinoma collected from a 69-year-old Caucasian woman with pleural effusion. It is a differentiated mammary epithelium which has many features such as estradiol processing ability through cytoplasmic estrogen receptors. MCF-7 consists of lumen cells that look and are shaped more differently. Cancer cell lines show characteristics of primary tumors with their characteristics such as copy number and gene expression. One of the concerns regarding cell lines is the maintenance of stability in genomic and expression patterns over multiple passages. However, it has been proven that cell lines do not accumulate abnormalities recurring over time [6].

MATERIALS AND METHODS

The study was carried out at Dicle University Science Technology Application and Research Center Laboratory. The sample was centrifuged at 4,000g for 30 minutes at 15°C for degreasing. After degreasing, 1M NaOH was added and the pH was adjusted to 7.6. Then, whey protein was obtained from the supernatant by centrifugation at 4,000g for 30 minutes at 15°C. Whey protein was passed through a 0.22 um microfiltration membrane filter using a Millipore branded vacuum pump to remove microorganisms. Frozen samples brought to the Analytical Chemistry Laboratory of Dicle University Faculty of Pharmacy were dried in a lyophilizer of Christ Freezone brand. Lyophilization or freeze-drying is the removal of water from frozen samples by applying 0.070 hPa pressure at -50°C. Whey protein was completely powdered after 96 hours of lyophilization.

Preparation of cell lines

DMEM (Sigma-Aldrich, USA) medium containing 2.2 g/L sodium bicarbonate was used for Mcf-7 cell line. It was centrifuged at 3,500 RPM for 6 minutes. Mcf-7 cell culture medium was transferred to sterile cell growth vessels (flasks) of 25 cm^2. The flask was placed in an incubator containing 5% carbondioxide at 37°C. The flasks were checked daily under an inverted microscope, the media thereof were changed, and when they became confluent, they were passaged and multiplied.

Figure 1: Breast Cancer Cell (MCF-7), Inverted Microscope (40x)

MTT TEST

In our study, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test, which is one of the cell viability assays, was preferred and applied. The basic principle of the MTT assay is based on the reduction of MTT to formazone crystals by live cells and is applied to determine mitochondrial activity. At a volume of 90 µl with a cell count of $3x10^3$ cells per mcf cell, 10 µl was added to 96-well plates in equal amounts to each well by serial dilution to receive 6,400 µg/ml, 3,200 µg/ml, 1,600 µg/ml, 800 µg/ml and 400 µg/ml doses of whey protein. No sample was added to the control well. Then, they were left in the incubator for different periods such as 24, 48 and 72 hours. After incubation, 10 µl MTT solution (5 mg/ml) was added and kept in a 37°C incubator with 5% CO_2 for 3 hours. After the incubator, 100 µl DMSO was added to the wells with a multiple pipette to dissolve the formazone crystals and the 96-well plate was covered with aluminum foil and shaken for 10 minutes. At the end of this period of time, absorbance was measured in a microplate reader (570 nm). The envisaged protocol was applied at 48 and 72 hours and absorbance values were obtained.

Determination of IL-2, IL-6 and Tumor Necrosis Factor Alpha (TNF-α) cytokine levels

The determination of cytokine levels in cows' whey sera was performed with Sandwich ELISA" (Kit No 202011017) test kits. The kits provide the necessary reagents to detect endogenous levels of cytokines based on the principle of survival by solid-phase sandwich

enzyme-linked immunosorbent assay (ELISA) and are used for this purpose.

Statistical Analysis

Quantitative data obtained in the study were expressed as arithmetic mean ± standard deviation with post-hoc analysis. The results of the graphs related to the study were obtained with Graphpad Prism 8 program (GraphPad Software, http://www.graphpad.com). Statistical analysis of the MTT study was performed using SPSS 22 software. The significance level in different groups was determined as p< 0.05.

3. RESULTS

The mean value of cow colostrum whey protein in Mcf-7 cancer cells in control group and different concentrations for 24 hours, the control group being 100±0.0, were 93.05±7.18 at 400 µg/mL; 52.73±7.85 at 800 µg/mL (p<0. 001); 48.69±6.89 at 1,600 µg/mL (p<0.001); 39.93±4.97 at 3,200 µg/mL (p<0.001); 37.06±7.74 at 6,400 µg/mL (p<0.001), and it was determined that there was a significant difference between the control group and the trial group (p<0.001). The statistical difference between 6,400 µg/mL and 400 µg/mL (p<0.001); 800 µg/mL (p<0.05) and 1,600 µg/mL concentrations is significant (p<0.05). The statistical difference between 6,400 µg/ml and other concentrations is highly significant (p<0.001). The statistical difference between 3,200 µg/ml and other concentrations is highly significant (p<0.001).

With the control group having the value of 100 ± 0.0, the mean value for 48 hours was 94.55 ± 4.63 at 400 µg/mL; 92.09 ± 4.82 at 800 µg/mL; 87.38 ± 5.55 at 1,600 µg/mL (p<0.05); 67.23 ± 9.74 at 3,200 µg/mL (p<0.001) and 43.52 ± 6.41 at 6,400 µg/mL (p<0.001), and a significant difference was determined between the control group and the study group. The statistical difference between the concentrations of 6,400 µg/mL and 400 µg/mL was highly significant (p=0.001), and in addition, the statistical difference between the concentration of 6,400 µg/mL and other concentrations was highly significant (p<0.001).

With the control group having a mean value of 100±0.0, the mean value for 72 hours was 92.15 ± 2.67 at 400 µg/mL, 83.36±7.08 at 800 µg/mL (p=0.001), 78.11±6.61 at 1,600 µg/mL (p<0.001), and 63.31 ± 10.3 at 3,200 µg/mL (p<0.001) which showed a significant difference and 45.78 ± 5.08 at 6,400 µg/mL (p<0.001) which also indicated a significant difference. The statistical difference between 6,400 µg/ml and other concentrations was highly significant (p<0.001). The statistical difference between 3,200 µg/ml and other concentrations is highly significant (p<0.001).

Table I: Cow Whey MCF-7 Findings

	24h		48h		72h	
	Mean ±SD	P Value	Mean ± SD	P Value	Mean ± SD	P Value
NT	100±0.00^{a,2}		100±0.00^{ac,1,2}		100±0.00^{ab,3,2}	
400µg/ml	93.05±7.18^{b,1}	p>0.05	94.55±4.63^{bc,2}	p>0.05	92.15±2.67^{b}	p>0.05
800µg/ml	52.73±7.85^{ab,2,1}	P<0.001	92.09±4.82^{c,2}	p>0.05	83.36±7.08^{ab,3}	p=0.001
1,600µg/ml	48.69±6.89^{ab,2,1}	p<0.001	87.38±5.55^{ac,1,2}	p<0.05	78.11±6.61^{ab,2}	p<0.001
3,200µg/ml	39.93±4.97^{ab,2}	p<0.001	67.23±9.74^{ac,2}	p<0.001	63.31±10.3^{ab,2}	p<0.001
6,400µg/ml	37.06±7.74^{ab,2}	p<0.001	43.52±6.41^{ac,2}	p<0.001	45.78±5.08^{ab,2}	p<0.001

^{a,b,c} Significance level differences between absorbances in the same column are indicated by the same letters.
[1]The statistical difference between absorbances with the same number in the same column is significant (p<0.05).
[2]The statistical difference between absorbances with the same number in the same column is highly significant (p<0.001).
[3]The statistical difference between absorbances with the same number in the same column is highly significant (p=0.001).

In MCF-7 cancer cell line, IC50 value for cow whey at 24 h was 5.605 µg/ml, R^2 = 0.968; IC50 value at 48 h was 5.257 µg/ml, R^2 = 0.976, and IC50 valu e for cow whey at 72 h was 3.144 µg/ml, R^2 = 0.373.

MCF-7 cell viability rate in cow whey

Cell viability rates of cow whey protein in MCF-7 cells (with control being 100%) depending on dose and time were for 24h, respectively: 92.15% at 400 µg/ml; 83.36% at 800 µg/ml; 78.11% at 1,600 µg/ml; 67.02% at 3,200 µg/ml, and 45.57% at 6,400 µg/ml. For 48h they were determined to be 94.55% at 400 µg/ml; 92.09% at 800 µg/ml; 87.38% at 1,600 µg/ml; 67.23% at 3,200 µg/ml, and 43.52% at 6,400 µg/ml. As for 72h, they were determined to be 93.05% at 400 µg/ml; 52.73% at 800 µg/ml; 48.69% at 1,600 µg/ml; 39.93% at 3,200 µg/ml, and 37.06% at 6,400 µg/ml (Figure 1).

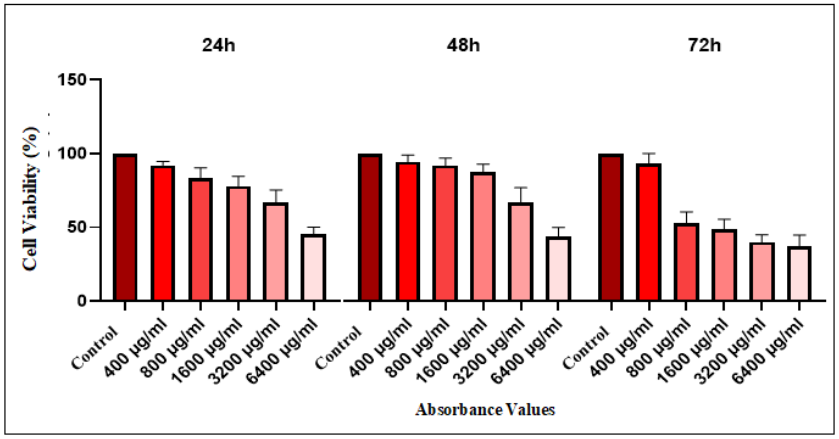

Figure 2: Cell viability rates determined by MTT in cow whey MCF-7 cells

IL-2, IL-6 and Tumor Necrosis Factor Alpha (TNF-α) Cytokine Levels

The interleukin family of cytokines is released by T-lymphocytes and macrophages. Interleukin helps maturation of B-lymphocytes and stimulates differentiation of lymphocytes. Interleukin accelerates immunoglobulin metabolism in B-lymphocytes. Sandwich ELISA assay was used to determine cytokine levels in whey proteins. IL-2 levels of whey protein were 136.52±6.82 with $p<0.001$ in cow whey of 6,400 µg/ml whereas 191.19±7.03 with $p<0.05$ in cow whey of 3.200 µg/ml.

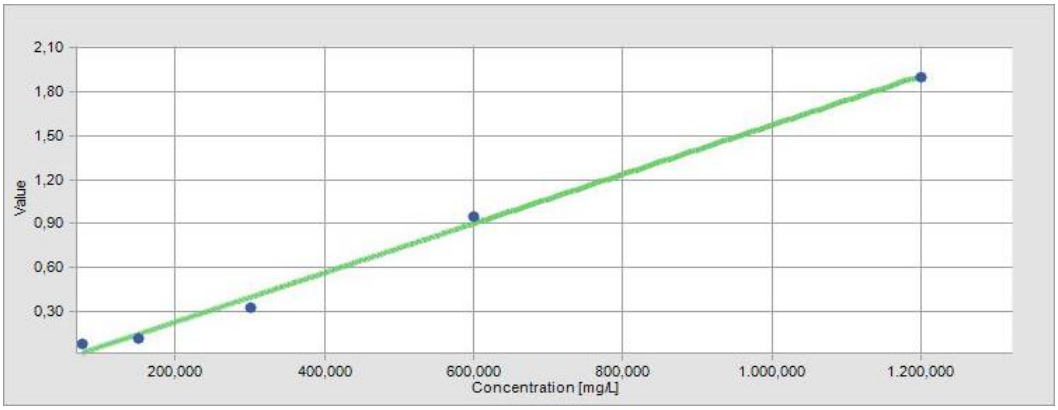

Figure 3: Standard Curve Drawing of cow whey IL-2

In the study, IL-6 levels were calculated at concentrations ranging from 3,200 µg/ml to 6,400 µg/ml, which showed a high level of cytotoxic effect among different concentrations of whey protein. In our group, the highest IL-6 levels in cow whey were at 6,400 µg/ml with 46.95±4.11 and $p<0.001$ as well as cow whey at 3,200 µg/ml with 60.55±3.04 and $p<0.001$.

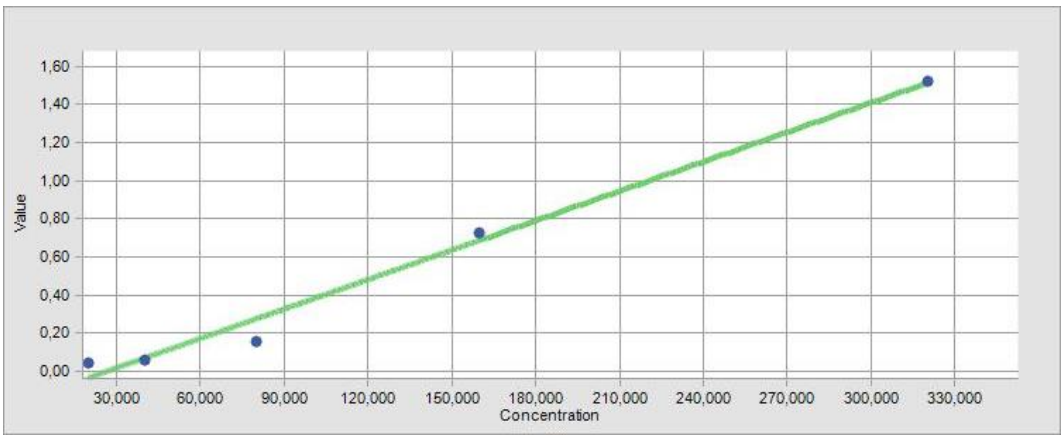

Figure 4: Standard Curve Drawing of cow whey IL-6

Tumor Necrosis Factor-α levels were calculated at concentrations ranging from 3,200 µg/ml to 6,400 µg/ml, which showed a high level of cytotoxic effect among different concentrations of whey protein. The highest Tumor Necrosis Factor-α levels in our group were found in cow whey 6,400 µg/ml 43.34±0.39 p<0.001, and cow whey 3,200 µg/ml 54.86±0.83 p<0.001.

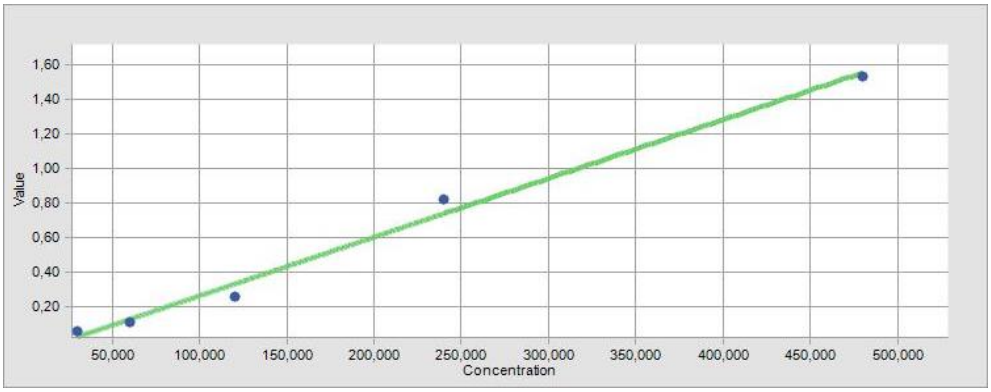

Figure 5: Cow whey TNF-α Standard Curve Drawing

4. DISCUSSION

Cancer is a disease in which the cells in the body proliferate uncontrollably in an abnormal way and spread into the body, causing metastasis. In short, cancer is a disease that occurs following the disruption of the mechanisms that control the uncontrollable proliferation of cells [7]. The most common type of cancer in women in the world is estimated to be breast cancer [8]. The breast is composed of milk glands and milk ducts that regulate milk production in women. The uncontrolled proliferation of cells in these milk glands and ducts in the breast tissue and their metastasis to different parts of the body is referred to as breast cancer. It is estimated that one in eight women will be diagnosed with breast cancer in her lifetime and one in thirty women will die from breast cancer [9]. Cancer treatment includes surgery, chemotherapy, radiotherapy and immunotherapy [10]. For instance, chemotherapy, which is often the treatment of choice for cancers in general, can also have a negative impact on healthy cells by causing toxicity in normal cells, resulting in irreversible side effects that can sometimes last a lifetime. These drawbacks necessitate the need to develop treatment strategies and treatment adjuvants with minimal side effects [11].

Various studies have been conducted on whey and casein proteins in milk. In these studies, especially the effects of whey proteins have gained importance. McIntosh et al. arrived at the conclusion that proteins in dairy products, particularly the whey proteins, play an important role in prevention of cancers [12]. In our study, we found that that cow whey proteins killed 62.94% of cancer cells in 72 hours at the highest dose of 6,400 µg/ml in breast cancer.

In their study, Fakharany *et al*., showed that albumin protein in human, cow and camel milk inhibited tumor cells in a dose-dependent manner by using albumin-oleic acid complex against CaCO2, HepG-2, PC-3 and MCF-7 cells according to MTT method [13]. In this study, due to the presence of serum albumin in cow whey, the result that it showed cytotoxic activity against MCF-7 cells in a dose- and time-dependent manner according to MTT method supports the findings of Fakharany *et al*., 2018.

5. CONCLUSION

As a result, the antiproliferative activities of whey proteins were tested. The highest antiproliferative effect of whey protein against Mcf-7 cells was found at high doses of cow whey protein. These findings reveal that the anticancer activity is high in whey protein and this effect varies depending on the dose and time.

Conflict of Interest Statement: We declare that there is no conflict of interest between the authors.

Financial Support: This study was supported by the Scientific Research Projects Unit of Dicle University with the project numbered Veterinary 20.002.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

Financial Disclosure: This study was supported by the Scientific Research Projects Unit with the veterinary project no: 20.002.

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