Original Article

Effect of Cow Colostrum, Mare Milk, and Human Milk on the Viability of Lung Healthy and Cancer Cell Lines

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Abstract

This study investigated the effects of lyophilized mare milk, human milk, and cow colostrum on both human lung cancer cell line called A549, and healthy lung cell line called MRC5. Mare milk, human milk, and cow colostrum varieties were applied to 6 replicates in both cell lines with lyophilized milk concentrations ranging from 50 - 3200 ppm. The cell viability was monitored by optic microscopy and determined by the MTT test. ANOVA and Duncan's multiple range tests were used to analyze data. The results of this study indicated that the most effective milk type on reducing the A549 lung cancer cell line was human milk, followed by mare milk; however, cow colostrum showed little effect. It was observed that human milk and mare milk had anti-proliferative effects on lung cancer cell line at concentrations which were non-toxic to healthy lung cell line.

Keywords: Cell viability, Colostrum, Cow milk, Human milk, Mare milk

1. Background

Milk and dairy products from animals can be used in the treatment of cancer and various diseases. Cancer is the most common risk that threatens human health worldwide, and a total annual economic cost is approximately US$ 1.16 trillion (1,2). Lung cancer is the leading cause of cancer-related deaths worldwide (3), accounting for about 2 million deaths per year (4).

Milk is a highly nutritious food for both children and adults since it is a source of high-quality proteins, vitamins, and minerals. Milk proteins are good sources of bioactive peptides that provide a variety of biological activities including antioxidant, immune-modulating, cholesterol-lowering, anti-hypertension, anti-microbial, and anti-cancer (5-7). With these unique properties, milk originated from different mammals is being used in cancer studies (6,8,9).

Human breast milk contains a variety of bioactive agents that modify the immune system function, gastrointestinal tract, and neuronal development (10). The results of an increasing number of studies have indicated that human milk offers protection against breast cancer, ovarian cancer, type II diabetes, obesity, and rheumatoid arthritis (11-13). Colostrum is known as a nutrient-packed fluid produced by the mammary glands in the last period of pregnancy just before birth. It contains developmental, immune, and tissue repair factors (14,15). Lactoferrin (LF) protein, which is found in high amounts in the milk of mammals, has been shown that reduces non-small cell lung carcinoma (16). Akca et al. conducted a study on the effect of donkey milk on lung cancer lines (9). Koumiss is a traditional milk beverage and, - mildly alcoholic - , dairy-based drink that is sour-tasting produced from the fermentation of mares' milk (17,18). Traditionally, it was produced from the milk of horses by residents in Central Asia and China, which is one of the most important basic food products (19). There has been an increasing interest in the production of koumiss at the industrial level due to its biotechnological potential and its benefits to human nutrition and health (20-22).

Considering the content of the milk from different sources and their activity in cancer, it is still highly demanded to investigate the effect of milk on different types of cancer. In this study, the cell viability of cow colostrum, mare milk, and human milk was evaluated on the viability of lung healthy and cancer cell lines. To this end, the human lung cancer cell line, called A549, - and a healthy lung cell line, called MRC5, were employed.

2. Methods

2.1. Preparation of Milk Samples

Cow colostrum, mare milk, and human milk were used for this study. Mare milk was obtained from Alas Mare Farm, - Izmir Kemalpasa, - Turkey. Cow colostrum and human milk were obtained from Sinop, - Turkey. The milk samples were kept at 110 °C for 10 min and then stored at -55 °C in the freezer for 24 h. The milk samples were lyophilized at -80 °C for 2 days before the cell viability tests. Hydrogen peroxide (H_2O_2) was used as a positive control at a single concentration of 150 μM. Sterile distilled water

was used in all experiments.

2.2. Cell growth procedure

Healthy human lung fibroblast (i.e., MRC5) and human lung cancer (i. e., A549) cell lines were provided from the American Type Culture Collection (Manassas, USA). The A549 lung cancer cells were cultured in F-12K medium supplemented with 10% fetal bovine serum (FBS), penicillin – potassium (50 µg/ml), streptomycin sulfate (10,000 µg mL-1), amphotericin B (25 µg mL-1), and 1% L-glutamine. The MRC5 lung fibroblast cells were cultured in Eagle's minimal essential medium supplemented with 10% FBS, penicillin – potassium (50 µg/ml), streptomycin (10,000 µg mL-1), amphotericin B (25 µg mL-1), and 1% L-glutamine. Cells were grown and stocked until the cell density reached 80% in the incubator in 5% CO_2 and 90% humidity medium at 37 °C.

2.3. Application of Lyophilized Milk Samples to Cell Cultures and Determination of Cytotoxic Effects

When the cell density reached 85% in a flask, a 5-20x10^4 number was added in 96-well plates and kept for 24-48 h to reach the appropriate density for cytotoxicity experiments. Later, lyophilized milk samples were applied to the cells in the concentration range of 3200-50 ppm. Pasteurized and lyophilized milk samples were treated with 7 different dose ranges using a negative control and a positive control group. For negative controls, the cells were kept in the growth medium. In positive controls, hydrogen peroxide (H_2O_2) at 250 µM concentration was applied to the cells. Cytotoxic effects were determined by the MTT method. That indicates the mitochondrial activity. For this purpose, 5 mg/ml of MTT in 100 µl of growth medium was added to each well and incubated in an incubator at 37 °C for 4-12 h. Then the top liquid in the wells was discarded and 150 µl dimethyl sulfoxide was added to the wells to dissolve formazan crystals. To determine changes in cell proliferation, measurements were performed by using a microplate reader at 570 nm.

The cell viability of each set was calculated according to the given formula:

$$\% \text{ Cell Viability} = \frac{\text{Cell absorption with compound} - \text{Absorption of empty well}}{\text{Absorption of control cell} - \text{Absorption of empty well}} \times 100$$

Moreover, lyophilized milk samples were applied to the cells followed by being washed 3 times with phosphate-buffered saline before optic microscopy imaging.

2.4. Statistical Analysis

The data collected in this study were analyzed in SPSS software (version 15.1) using ANOVA and Duncan's multiple range tests for the numerical data obtained from the experiments.

4. Results

Mare milk, human milk, and cow colostrum samples were applied to the A549 lung cancer cell line and MRC5 healthy cell line. The cell viabilities of both A549 and MRC5 cell lines for different milk samples at different concentrations are summarized in Table 1.

Table 1 presents the effects of three types of milk namely, human, mare, and cow colostrum, in different concentrations on the A549 lung cancer cell line and MRC5 healthy lung cell lines. The mean and standard deviation scores and homogeneity groups (the codes used in Duncan's multiple range test) are tabulated in Table 1, which were acquired from 6 replicates in both cell lines.

For the case of mare milk, in both cell lines, dose-dependent cytotoxicity was detected in a high concentration range (Figure 1). For all concentrations, MRC5 showed higher cell viability than A549. Even at 3,200 ppm, the cell viability values were still about 50%.

The employment of cow colostrum resulted in around 20% in both A549 and MRC5 cell lines at

Table 1. Results of Experimental Tests and Duncan test for various milk effects on lung cancer

Concentrations (ppm)	Cell type	Human Milk			Mare Milk			Cow colostrum		
		Mean	SD	HG	Mean	SD	HG	Mean	SD	HG
50	A549 cancer lung cells	0.40966	0.00079	GH	0.58466	0.029762	LM	0.612	0.0051215	MN
	MRC5 healthy lung cells	1.29933	0.01700	Z	1.01933	0.0057726	VW	0.86966	0.0016127	U
100	A549 cancer lung cells	0.38366	0.01905	FG	0.53666	0.060722	K	0.58	0.020976	LM
	MRC5 healthy lung cells	1.19833	0.00762	Y	0.826	0.0423509	T	0.76266	0.003726	QR
200	A549 cancer lung cells	0.35566	0.00854	F	0.484	0.03234	J	0.591	0.0028284	LM
	MRC5 healthy lung cells	1.14666	0.00310	X	0.817	0.0124096	ST	0.73266	0.023390	Q
400	A549 cancer lung cells	0.34933	0.01156	EF	0.459	0.025349	IJ	0.55666	0.0027283	KL
	MRC5 healthy lung cells	1.05366	0.00610	W	0.78233	0.0624362	RS	0.639	0.0171230	NO
800	A549 cancer lung cells	0.26566	0.01256	C	0.416	0.078153	GH	0.53666	0.003212	K
	MRC5 healthy lung cells	1.055	0.007	W	0.733	0.0386626	Q	0.59466	0.0085128	LM
1600	A549 cancer lung cells	0.25133	0.02178	C	0.43266	0.042603	HI	0.37033	0.001526	F
	MRC5 healthy lung cells	1.00833	0.00100	V	0.67933	0.0114486	P	0.538	0.0089218	K
3200	A549 cancer lung cells	0.07933	0.00981	A	0.353	0.0492869	F	0.173	0.0054037	B
	MRC5 healthy lung cells	0.28333	0.01040	CD	0.65666	0.0092096	OP	0.314	0.0032863	DE

Notes: SD: Standard deviation, HG: Homogeneity group: a section of experimental units comparable to each other in terms of a checked feature. Different letters in the columns show statistical differences, and the same letters in the columns represent that there is no statistical difference between samples according to Duncan's test of the multiplication range at 95% confidence level in SPSS. While groups with more than one letter and with a common letter show no statistically significant difference, groups that lack a letter in common are statistically different.
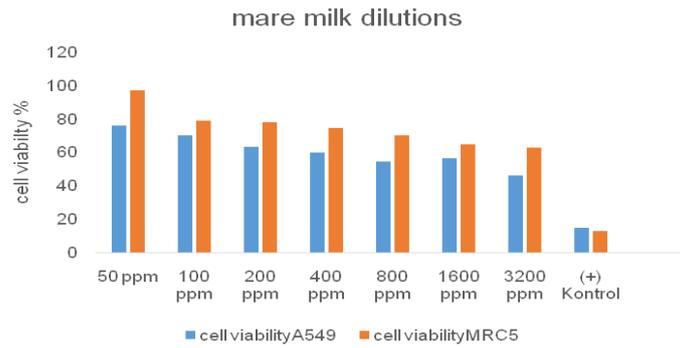
Figure 1. Effect of mare milk on cell viability of A549 and MRC5 cell lines at different concentrations

3,200 ppm (Figure 2). Similar to mare milk, dose-dependent cytotoxicity was observed in a high concentration range. In most cases, A549 cells showed higher viability than MRC5 cells.

The employment of human milk remarkably decreased the cell viability of A549 cell lines. However, the cytotoxicity in the MRC5 cell line was distinctively higher indicating the biocompatibility of human milk on this cell line. The cell viability started from around 120% and decreases to about 25%. It is clear that the interaction of human milk with MRC5 cells significantly improved cell viability.

The objective images in figures 4a and 4b are the microscope pictures of mare milk (shown in Figure 1 and Table1) applied to A549 cancer cells and MRC5 healthy cells at concentrations of 3,200 ppm and 50 ppm, respectively.

The objective image in Figure 5a is a microscope picture of the interaction of MRC5 healthy cells with the medium as a negative control. The microscopic picture in Figure 5b is an image of human milk (shown in Figure 3 and Table1) applied to A549 cancer cells at a concentration of 1,600 ppm to reduce cell viability.

The objective images in figures 6a and 6b are the microscope pictures of human milk (shown in Figure 3
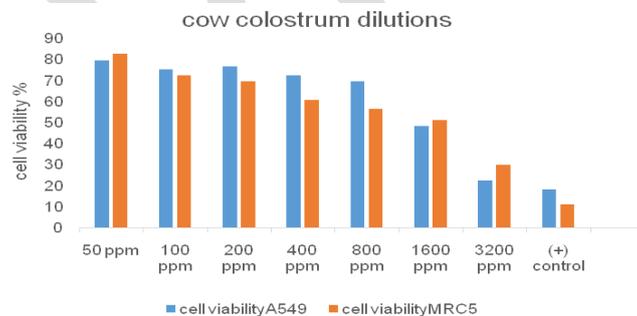
Figure 2. Effect of cow colostrum on cell viability of A549 and MRC5 cell lines at different concentrations
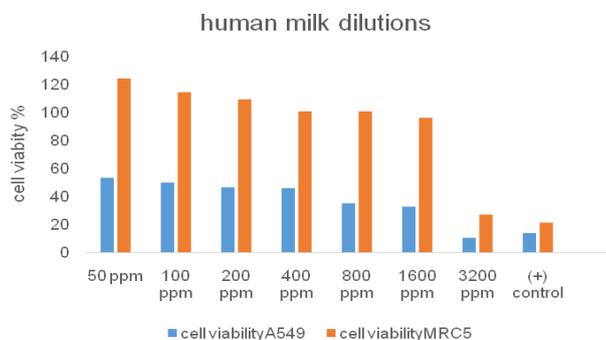
Figure 3. Effect of human milk on cell viability of A549 and MRC5 cell lines at different concentrations
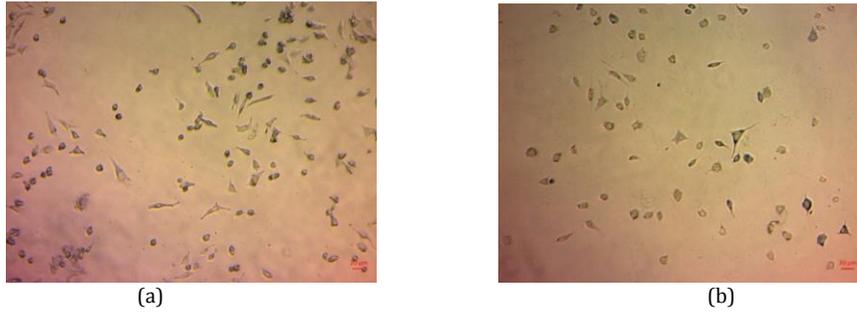
Figure 4. Optic images of mare milk exposed to cell lines; (a) A549 cancer cell line (3,200 ppm) and (b) MRC5 cell line (50 ppm); The images were collected at 10X magnification.

Figure 5. Optic images of mare milk and human milk exposed to cell lines; (a) Mare milk exposed to MRC 5 cancer cell line (1,600 ppm) and (b) human milk A549 cell line (1,600 ppm); The images were collected at 10X magnification

and Table1) applied to A549 cancer cells and MRC5 healthy cells at concentrations of 3,200 ppm and 100 ppm, respectively.

The objective image in Figure 7a is a microscope picture of human milk (shown in Figure 3 and Table1) applied to MRC5 healthy cells at a concentration of 200 ppm. The objective image in Figure 7b is a microscope view of the interaction of A549 cancer cells with the medium as a negative control.
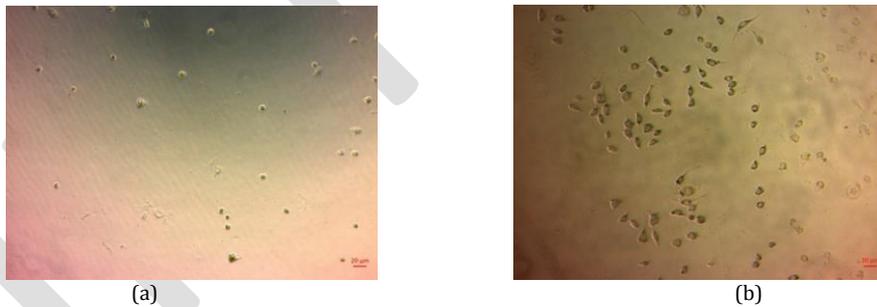
Figure 6. Optic images of human milk exposed to cell lines; (a) A549 cancer cell line (3,200 ppm) and (b) MRC5 cell line (100 ppm); The images were collected at 10X magnification
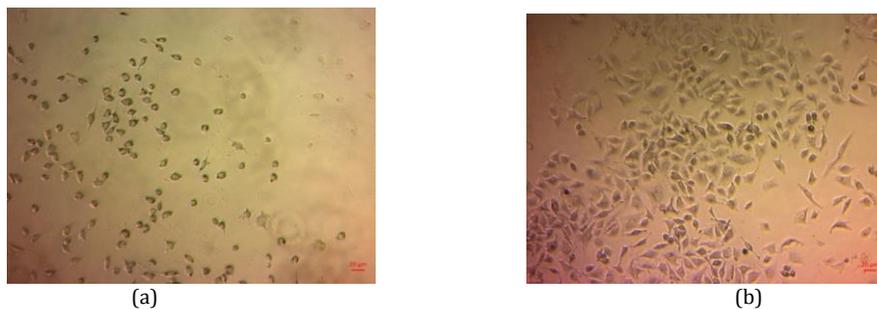
Figure 7. Optic images of human milk exposed to cell lines; (a) MRC5 cancer cell line (200 ppm) and (b) A549 cell line positive control; The images were collected at 10X magnification
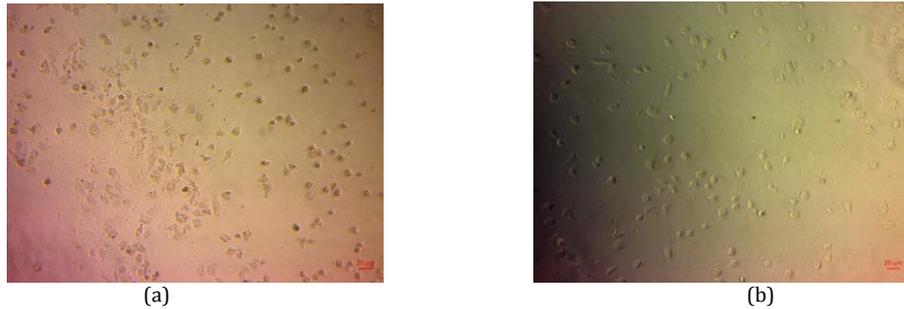
Figure 8. Optic images of positive control cell lines; (a) A549 cancer cell line and (b) MRC5 cell line; The images were collected at 10X magnification.
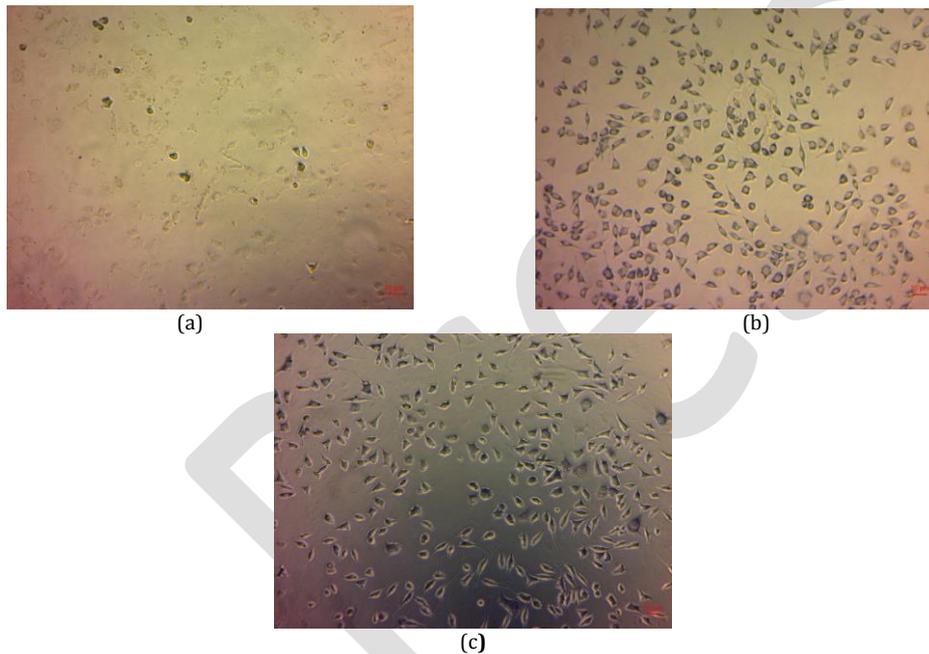
Figure 9. Optic images of cow colostrum exposed to cell lines; (a) A549 cancer cell line (3,200 ppm), (b) MRC5 cell line (50 ppm), and (c) MRC5 negative control; The images were collected at 10X magnification.

Figures 8a and 8b are the microscope views of the interactions of A549 cancer cells and MRC5 healthy cells with H_2O_2 as positive controls, respectively.

The objective images in figures 9a and 9b are the microscope pictures of cow's milk (shown in Figure 2 and Table 1) applied to A549 cancer cells and MRC5 healthy cells at concentrations of 3,200 ppm and 50 ppm, respectively. The picture in Figure 9c is a microscope image of the interaction of MRC5 healthy cells with the medium as a negative control.

5. Discussion

Milk products contain a large number of probiotic bacteria and metabolites. The release of this content during the fermentation of probiotic bacteria in dairy products may prevent colorectal carcinogenesis (23). Earlier reports showed that fermented milk products had many health-improving effects, such as the reduction of serum cholesterol, improvement of lactose metabolism, and reduction of cancer risk (24-26). Moreover, van't Veer et al. hypothesized that high consumption of fermented milk products (predominantly yogurt and buttermilk) may create protection against breast cancer (27).

The results of previous studies have shown the preventive effects of cow milk and its effect on breast and esophageal cancer in cell lines. However, in our study, lyophilized cow colostrum showed little effect on reducing lung cancer cells and improving the viability of healthy cells (Figure 2, Figure 9a, and Figure 9b). Duarte et al. (8) conducted a study on the anti-cancer properties of bovine milk lactoferrin on human breast cancer HS578T and T47D cells. The cancer cells were treated with lactoferrin concentrations ranging from 0.125-125 µM and they showed that LF has great potential to be used in breast cancer treatment (8). Farziyan et al. reported that LF obtained from bovine colostrum showed an inhibitory effect on
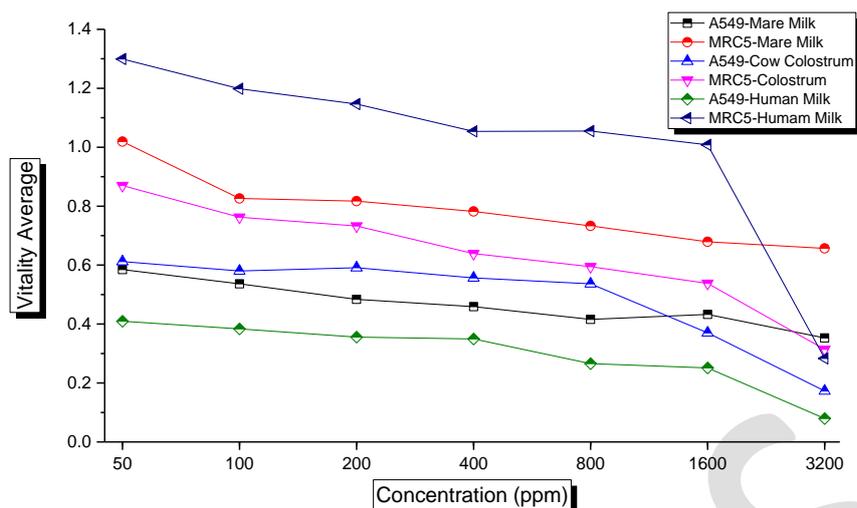
Figure 10. Concentration-dependent vitality average change graph of the effect of three milk groups on A549 and MRC5 cell lines.

esophageal cancer cell line KYSE-30, and the MTT assay test ([28]) indicated that 500 μg/ml of LF reduced cell viability in esophageal cancer cell lines KYSE by 53% and 80% after 20 and 62 h, respectively ([29]).

Furthermore, in another report, the effect of donkey milk on A549 lung cancer and BEAS-2B healthy lung cell lines were investigated. Donkey milk showed lower cytotoxic effects against healthy lung cell lines in comparison to the tumor cell line. This data indicated that donkey milk had anti-proliferative effects on lung cancer cells at concentrations that were non-toxic to normal lung cells ([9]). In our study, mare milk showed a similar effect in the same cancer lines (Figure 1 and Figure 4).

Figure 10 shows the variations in human milk, cow colostrum, and mare milk in A 549 cancer and MRC 5 healthy cell lines. Lyophilized human milk has been applied to A549 cancer cell lines and MRC5 healthy cell lines. The effect of cell viability was mostly detected in human milk (Table 1, Figure 5b, Figure 6a, Figure 6b, and Figure 7a). A similar change was observed by lyophilized mare milk at slightly lower values (Table 1 and Figure 4). Lyophilized cow colostrum showed little effect on reducing lung cancer cells and improved the viability of healthy cells (Table 1, Figure 2, Figure 9a, and Figure 9b). This change was found to be at its highest and lowest concentrations at 50 and 800 ppm, respectively. The most effective change in A549 cancer cell lines, compared to MRC5 healthy cell lines, was revealed to be in human milk (Figure 3, Figure 5b, Figure 6a, Figure 6b, and Figure 7a). In this case, it was observed that lyophilized human milk reduced the density of A549 lung cancer cells in parallel with increasing concentration; nevertheless, it increased cell viability in MRC5 healthy lung cells.

6. Conclusion

This study investigated the anti-cancer properties of various mammalian milk in 7 different concentrations by using A549 lung cancer cell line and MRC5 healthy lung cell lines as in vitro studies. Based on the results of this research, human milk was the most effective type of milk in reducing the human lung cancer cell line called A549, followed by mare's milk, while cow colostrum had little effect. Human milk and mare's milk have been observed to have anti-proliferative effects on the lung cancer cell line at concentrations non-toxic to the healthy lung cell line. The researchers of the current study will focus on the employment of these milk samples in animal models in their prospective study.

Footnotes

Conflicts of Interest: The author declare that there is no conflict of interest.
Ethical Approval: Ethical committee was not required for conduct this study in Turkey.
Funding/Support: The author declare that there is no funding.
Financial Disclosure: The author declare that there is no financial disclosure.
Informed Consent: Informed consent was not required to conduct this study in Turkey.

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