CHAPTER 5

Anti-cancer property of camel milk proteins and associated cytotoxicity mechanisms

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5.1 Introduction

Since millions of years, milk has evolved as a major source of bioactive molecules that provide nutrition, promote overall growth and prevent several diseases including cancer. Camel milk has been used for the treatment of multiple diseases such as diabetes, infant diarrhea, hepatitis, allergy, lactose intolerance and alcohol induced liver damage. It's therapeutic potential against many diseases including cancer has been reviewed (Dubey et al., 2016) and its benefits attributed to the presence of many immunologically important molecules such lysozymes, lactoferrin, lactoperoxidase, serum albumin, whey acidic protein, peptidoglycan recognition protein, small peptides, etc. Moreover, camelid antibodies, also present in milk, are very special and often referred to as nanobodies due to their small size. Although these are single domain antibodies (SdAb) containing only the heavy chain and not the light chain but they retain their specificity and stability (Desmyter et al., 2001). Therefore, the special features of these antibodies and the presence of other biologically important molecules and/or their derivatives confer camel milk with unique medicinal properties (Abdel Gader and Alhaider, 2016).

Cure of cancer still remains a worldwide challenge. The side effect caused by the present treatment modalities often limit patient compliance and make treatment a very challenging task. It is therefore necessary to explore effective alternate treatment modalities devoid of any side effect.

Camel milk has been traditionally used for cancer prophylaxis and treatment in Middle Eastern countries. Camel milk proteins are highly thermostable besides being resistant to acid hydrolysis. The scientific basis of the anti-cancer property of camel milk remains largely unexplored.

5.2 Outline of work

To begin with, the protein quantity was estimated from the milk collected and its protein was profiled by SDS-PAGE. The present study further explores the effect of semi purified camel milk fraction on viability of HeLa cells, their *in vitro* cell migration upon treatment. Also the ability of the whey fraction to induce DNA fragmentation has been looked into. Furthermore, the caspase-3 activity of camel milk treated cells has also been studied.

5.3 Results

5.3.1 Quantitation of camel milk protein

The protein content of the milk collected from five lactating camels are given in Table 8. The mean protein content and standard deviation was 32.04 and 7.32 respectively. Also given alongside are the lactation period of these camels. These values compare well (32.04 vs 32.2 g/l) with biochemical diversity of camel milk related studies done by others. Milk collected from camel 2 was used for further studies because of its most optimal protein content.

Table 7: Protein content of camel milk samples

Camel Number	Lactation Time (In months)	Protein content (mg/ml)
1	2	60.74
2	1.5	30.04
3	9	21.36
4	2	23.36
5	8	24.69

Mean = 32.04 and ; SE = 7.32

5.3.2 Profiling of camel milk proteins by SDS-PAGE

The protein profile of the milk samples collected from the five lactating camels was done by silver staining. The silver stained gel showed the presence of about ten proteins in all the samples as shown in Figure 6. The major one of these proteins in camel milk are lactoferrin, serum albumin, multiple immunoglobulins, various subtypes of caseins , TRAIL and α-lactalbumin. The same have been depicted in Table 6 (under section 4.2.3.1) along with their molecular weights, as cited in literature (Omar et al., 2016).

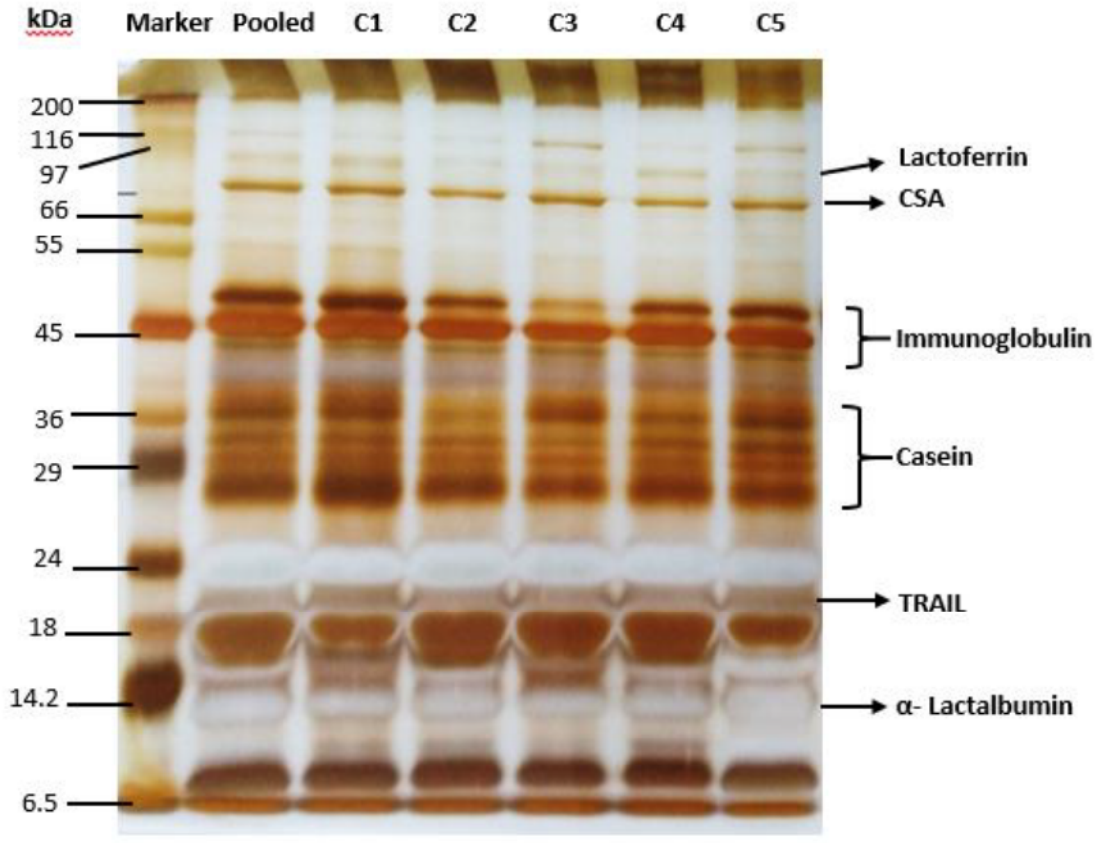


Figure 6: Protein profile of camel milk

5.3.3 Effect of camel milk, camel milk whey and Camel milk casein on HeLa cell viability

The ability of camel milk and its components to induce cytotoxicity in cancer cell lines, were tested by the MTT assay, The Figure 7 graph indicates comparison of percent viability of various dilutions of whole camel milk against HeLa cells for 24 and 48 hours interval. From the graph, it is clearly visible that for lowest concentration i.e. 5mg/ml of camel milk, there was no significant change in viability at 24h. At higher concentrations the cell viability declined in statistically significant manner. Further there was a concentration dependent decrease in viability after 48h in all the concentrations studied.

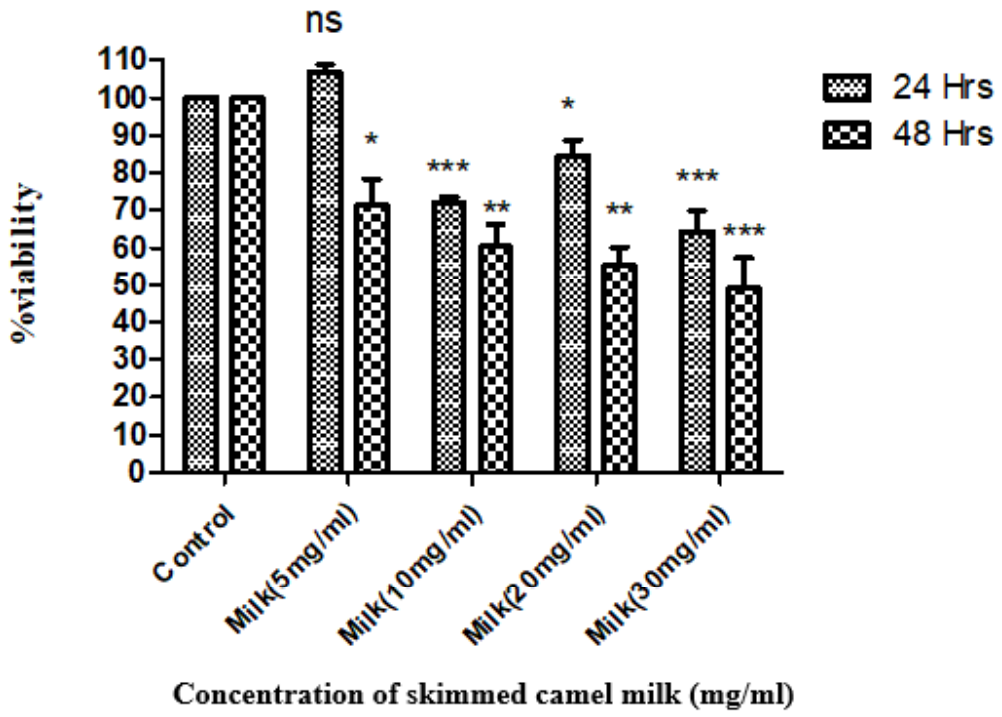


Figure 7: Percentage viability of defatted camel milk (at different protein concentrations) treated HeLa cells when compared with control (untreated HeLa cells)

The Figure 8 below shows the effect of camel milk casein on HeLa cells at various concentrations. It was observed that casein did not have a statistically significant impact on the viability of treated cells at any of the concentrations studied. Thus it can be inferred that camel milk casein is not cytotoxic to these transformed cells.

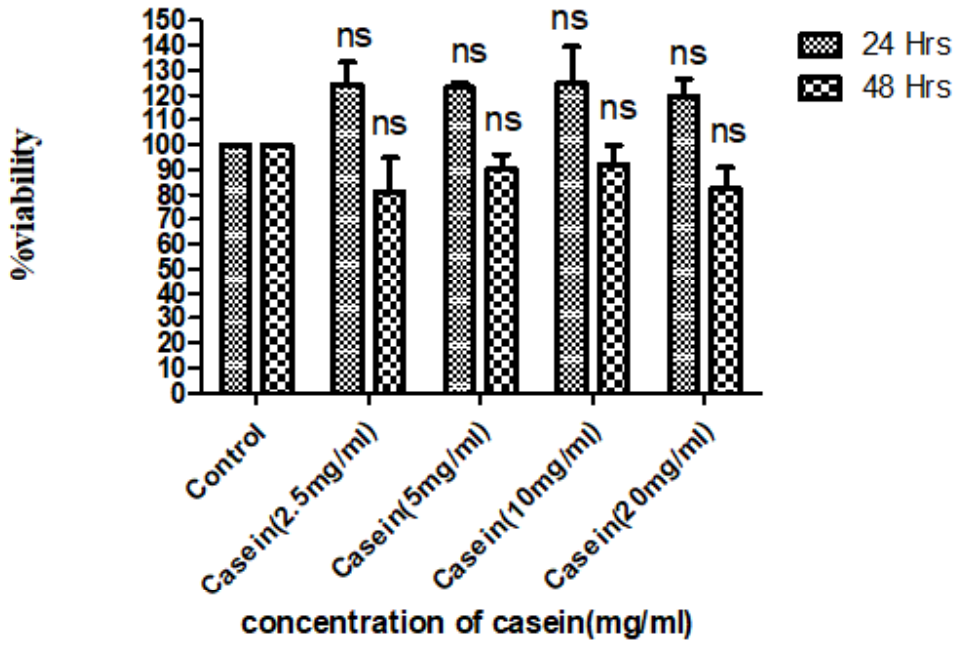


Figure 8: Percentage viability of camel milk casein treated HeLa cells

Unlike casein, the whey fraction of camel milk was found to be cytotoxic to these cells at 24h and 48h in a dose dependent manner as shown in Figure 9. This indicates that the cytotocoxicity of camel milk is primarily associated with the whey fraction only and it is even effective at 24h unlike whole camel milk which is effective only at 48h. Therefore only the whey fraction has been used for further studies at 24h.

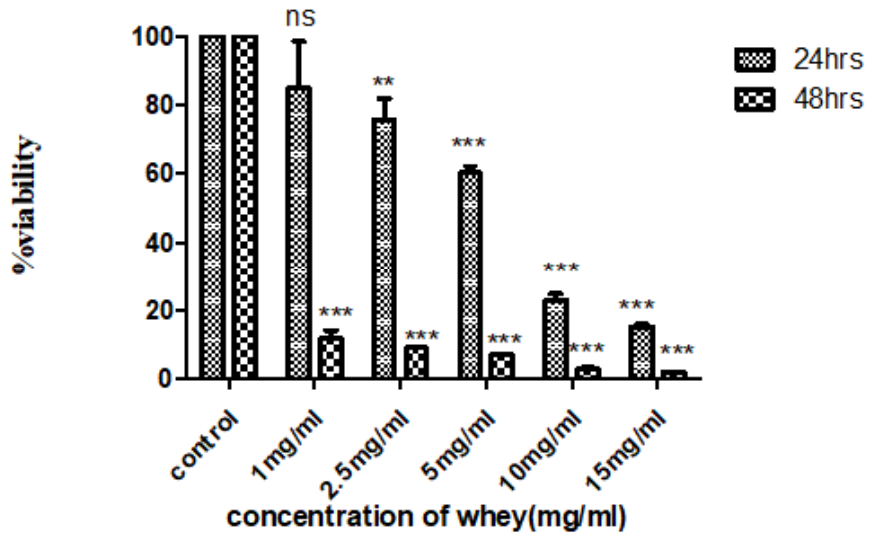


Figure 9: Percentage viability of camel milk whey fraction treated HeLa cells

In above figures 7, 8 and 9, Graph and statistical analysis was performed by using GraphPad prism5 software. Comparison among inter and intra groups was analyzed using one way ANOVA followed by Bonferroni multiple comparison test. $P< 0.05$ was considered to indicate statistically significant difference. Experiment was repeated thrice (n=3).

5.3.4 Cell migration assay

The scratch test was performed on camel milk whey treated cells to check the ability of whey proteins to inhibit migration of transformed cells *in vitro* taking cisplatin treated and untreated HeLa cells as positive and negative controls respectively. The figure below depicts migration of: untreated HeLa cells (uppermost row), HeLa cells treated with 4µg/ml (IC_{50}) of cisplatin (middle row), and HeLa cells treated with 7.5mg/ml (IC_{50}) of camel whey (lowermost row) after 24h and 48h respectively. It was observed that HeLa cells treated with whey at its IC_{50} of 7.5 mg/ml showed lesser migration than untreated cells after 24h. Cisplatin treated cells (at its IC_{50}) showed the least

migration. This result indicates the potential of camel milk whey in inhibiting migration of transformed cells from their primary site.

Cell Migration Assay (HeLa Cells)

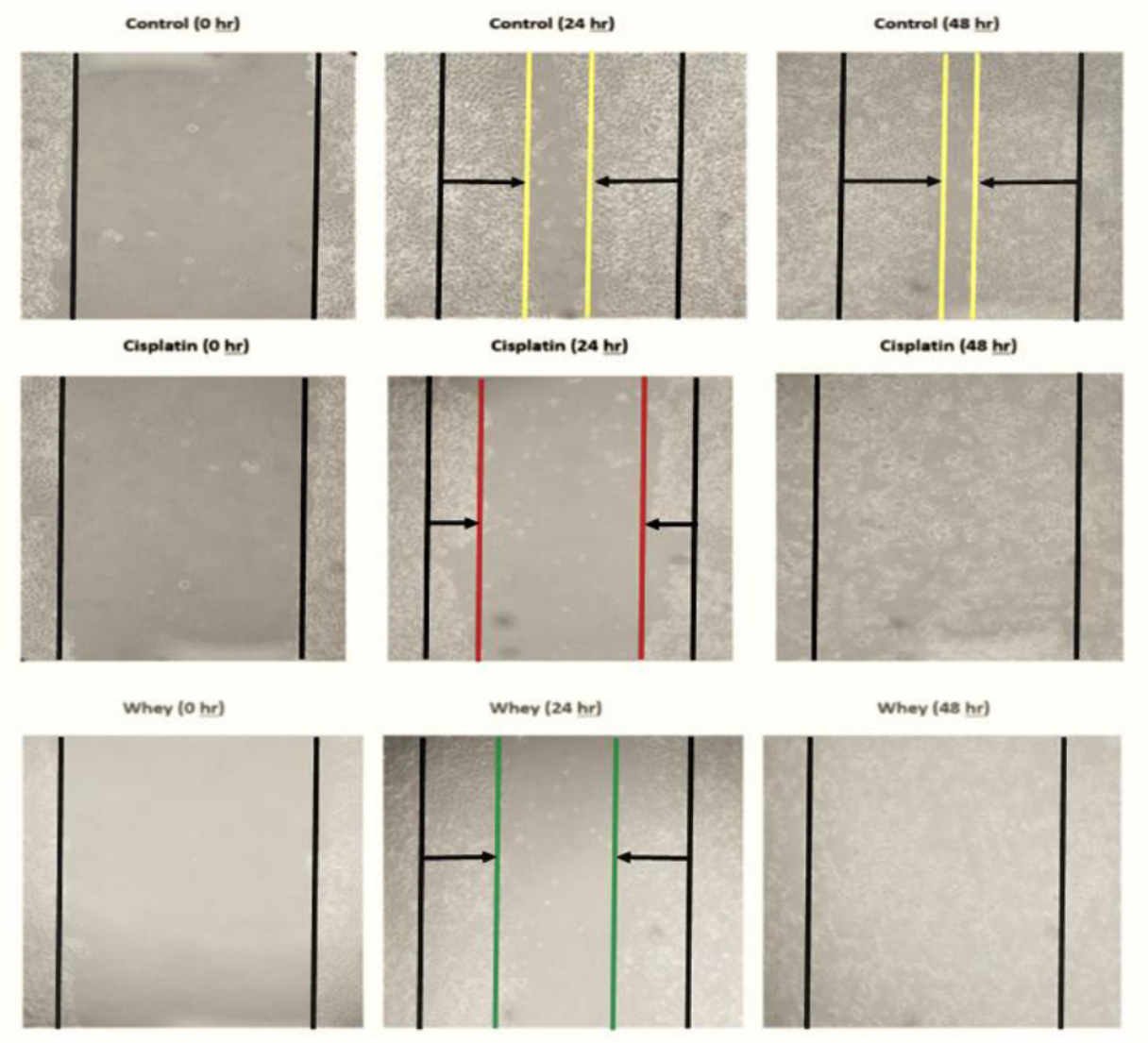
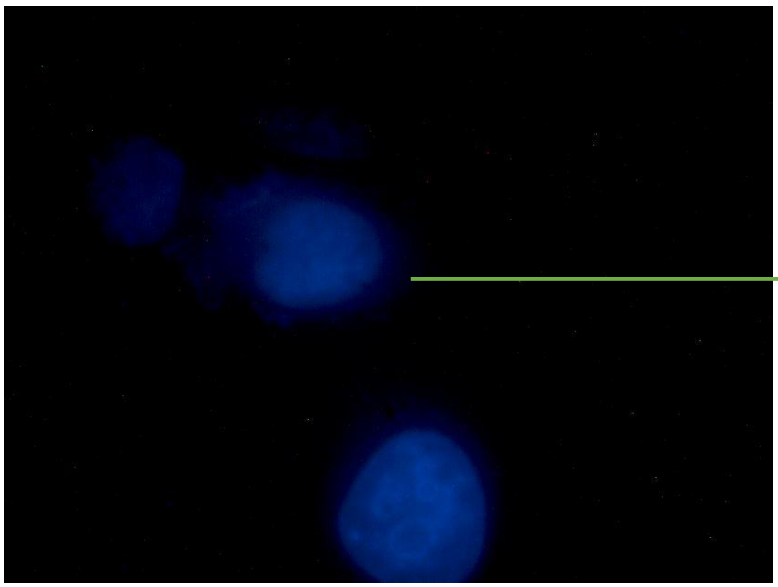


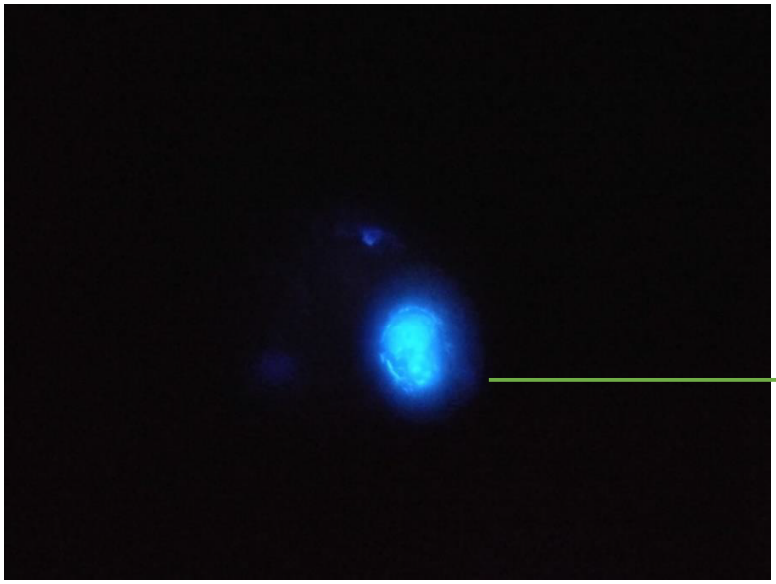
Figure 10: Effect of camel milk whey and cisplatin treatment on cells migration.

5.3.5 Effect of camel milk whey on HeLa cells studied by fluorescent microscopy

Camel milk whey treated cells were stained with DAPI and observed, the cells showed nuclear condensation and cellular shrinkage as shown in Figure 11 B. These features were more prominent at higher concentrations of whey treated cells. This was more prominent in cisplatin treated cells as compared to whey treated cells. Normal cells retained the usual morphology (Figure 11 A).

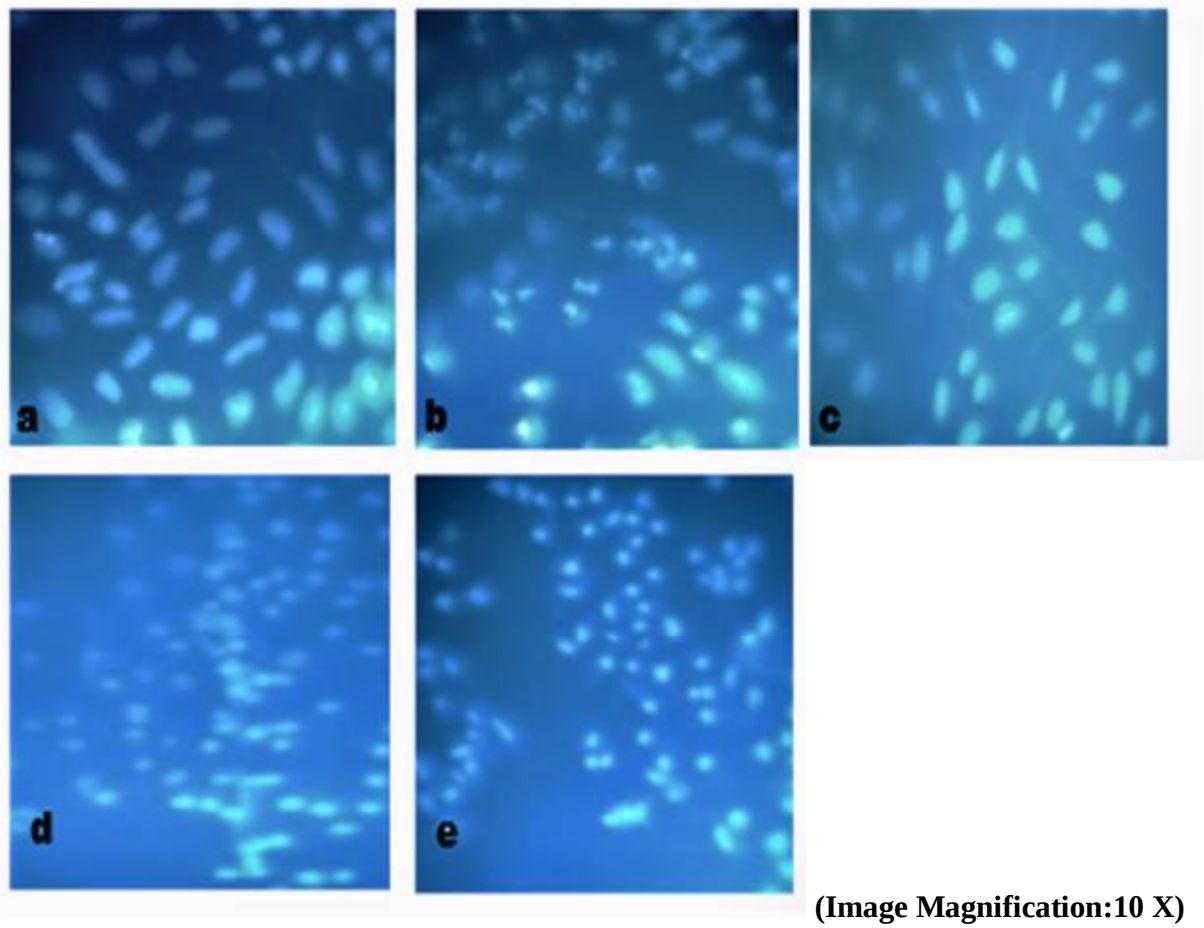


(Figure 11A)



(Figure 11B)

Figure 11A & 11B: Fluorescent microscopy of DAPI Stained HeLa cells without treatment (11A) and after 24h treatment with camel whey protein (11B) (Enlarged Images)



(Image Magnification:10 X)

Figure 11C: Fluorescent microscopy of HeLa cells after 24h of cisplatin and whey treatment

(a) Negative control (untreated cells); **(b)** Cells treated with IC_{50} (4µg/ml) of cisplatin; **(c)** Cells treated with low dose (5mg/ml) of whey; **(d)** Cells treated with- IC_{50} (7.5mg/ml) of whey; **(e)** Cells treated with high dose (10mg/ml) of whey.

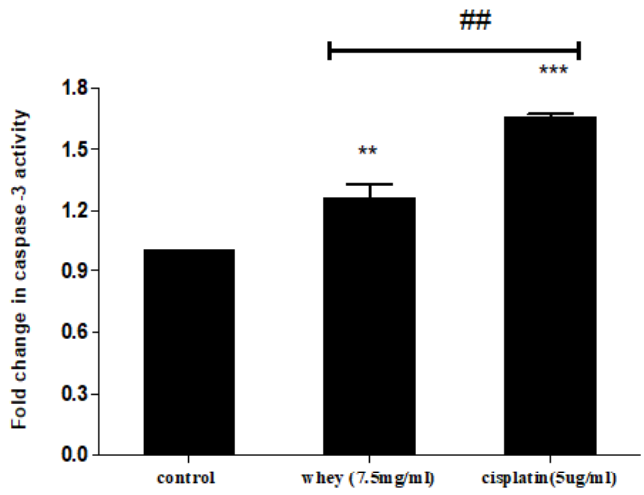


Figure 12: Caspase 3 assay of HeLa cells after treatment with IC_{50} of camel milk whey and cisplatin.

Graph and statistical analysis was performed by using GraphPad prism5 software. Comparison among inter and intra groups was analyzed using one way ANOVA followed by Bonferroni multiple comparison test. $P< 0.05$ was considered to indicate statistically significant difference. Experiment was repeated thrice (n=3).

Caspase – 3 Assay was performed on Hela cells with treatment of IC_{50} of Whey with respect to Cisplatin and control at 24 Hrs. There is 1.4 fold increase in caspase-3 activity in whey treated cells in comparison to control cells. Cisplatin shows a 1.6 fold increase. This suggests the ability of camel milk whey to induce caspase 3 activation in treated cells.

5.4 Discussion

This chapter looks into the various aspects related to the anti-cancer effect of camel milk. In the present study the anti-cancer property of camel milk was found to be associated with the whey fraction of camel milk but not its casein fraction. The whey fraction was subsequently investigated for its *in vitro* effect on cancer cell mobility by the cell migration assay. It has elicited an ability to inhibit cell migration. The treatment of the cervical cancer cell line, HeLa, with camel milk

whey showed its ability to induce nuclear condensation and cell shrinkage, which is a characteristic of apoptosis. It was also able to activate caspase-3, a vital enzyme involved in programmed cell death. In continuation to this chapter, the camel milk whey proteins have been discussed in detail in the next chapter.

In our study we observed significant cytotoxic potential of camel milk at high concentrations, but its whey was found to have far more cytotoxic potential in HeLa cells. Similarly, Korashy et al., 2012 also found camel milk to be cytotoxic against HepG2 and MCF7 cells. They also studied the effect of camel milk on the apoptotic signaling pathways in Human hepatoma HepG2 and Breast Cancer MCF7 cell lines. The study proved that whole camel milk treatment reduced the cell proliferation and also induced increased expression levels of effector caspase-3 mRNA in HepG2 cells. Similar effect was seen in the MCF7 cell lines also (Korashy et al., 2012b).

Besides whole milk some scientists have also studied anti-cancer property of camel whey protein hydrolysates. Hina Kamal et al., (2018) have reported an enhanced anti-proliferative, anti-diabetic and anti-inflammatory activities of camel whey proteins. This indicates its potential for utilization as bioactive and functional ingredient. Animal models for colon and mammary tumorigenesis have shown that whey proteins are better than other dietary proteins in suppressing tumor development (Parodi, 2007). These studies are indicative of the anti-cancer potential of whey proteins and their hydrolysates.

One of the strategies for protecting human cells and tissues from the toxic effects of carcinogenic and cytotoxic metabolites, includes attenuation of the carcinogen-activating genes, CYP1A1 signaling pathways. Several lines of evidence showed that induction of CYP1A1 is strongly correlated with increased incidence of human colon, rectal, and lung cancers (Shah et al., 2009; Slattery et al., 2004). Thus, CYP1A1 induction is considered a useful biomarker of exposure to

carcinogenic substances (Williams et al., 2000). New anti-tumor drugs like aminoflavone and benzothiazoles, require AhR-mediated signaling to induce DNA damage. This is a new treatment strategy for breast cancers with intact AhR signaling. Camel milk constituents are known to similarly act through aryl hydrocarbon receptor (Callero and Loaiza-Pérez, 2011). Accordingly, camel milk can be postulated to protect against or decrease the deleterious effects of many environmental toxicants and carcinogens such as PAHs, probably through modulation of AhR-regulated genes of CYP1A1 transcriptional and post transcriptional mechanisms.

We, by conducting cell migration assay, observed that whey treatment resulted in reduced the migration of HeLa cells. Similarly, Krishnankutty et al., (2018) have also studied the anti-cancer property of camel milk and its associated mechanism in colorectal and breast cancer cell lines. They too have observed cytotoxicity induced by camel milk in both cell types. They even observed a reduced migration induced in cancer cells. Furthermore, their work suggests autophagy as a mechanism of inducing cytotoxicity. The same authors in 2016 have demonstrated that Camel whey protein improves lymphocyte function and protects against diabetes in the offspring of diabetic mice.

Badawy et al., (2018) have reported the therapeutic effect of camel milk and its exosomes *in vivo* and *in vitro* in cancer. Administration of camel milk (orally) and its exosomes (orally and by local injection) decreased breast tumor progression was evident by increased apoptosis, higher DNA fragmentation, caspase-3 activity, *Bax* gene expression,. They also observed an inhibition of oxidative stress induction of apoptosis and inhibition of oxidative stress, inflammation, angiogenesis and metastasis in the tumor microenvironment.

Camel milk exerted anti-proliferative effects on human colorectal HCT 116 and breast MCF-7 cancer cells by inducing autophagy. Camel milk significantly reduced proliferation, viability as

well as migration of both these cells. The accumulation of LC3-II protein along with reduction in expression of p62 and Atg 5-12, the autophagy proteins implied induction of autophagy. The (GFP)-LC3 puncta (ring like structures) detected by confocal microscopy confirmed the autophagosome formation in response to camel milk treatment.

The mechanisms associated with camel milk and its fraction associated anti-cancer properties have evoked interest in recent times. Multiple caspases including caspase-3 are involved in the cytotoxicity mediated by programmed cell death. Many anti-cancer drugs act by activating the caspase group of enzymes. Henkels and Turchi, (1999) showed that Cisplatin-induced apoptosis proceeds by caspase-3 dependent and independent pathways in cisplatin-resistant and -sensitive human ovarian cancer cell lines. The results of Korashy et al., (2012) showed increase in caspase-3 mRNA levels by camel milk, which was completely blocked by the transcriptional inhibitor, actinomycin D; implying that camel milk increased *de novo* RNA synthesis. We have also observed increased activation of caspase-3 at the level of protein activation.

5.5 Conclusion

In this chapter it has been observed that camel milk is cytotoxic against HeLa cells only at a high concentration. The camel milk whey fraction is cytotoxic at all concentrations studied whereas the casein has not been found to be cytotoxic. Camel whey has showed the ability to inhibit migration of transformed cells. It was observed that whey enhances the activity of caspase-3 with respect to control.