DOI : 10.5958/2277-8934.2023.00007.3 *Vol 30 No 1, p 53-61*

CHROMATOGRAPHIC PURIFICATION OF PROTEINS WITH CYTOTOXIC POTENTIAL FROM CAMEL MILK WHEY AGAINST CERVICAL CANCER CELL LINE

Neelam Mahala, Aastha Mittal, Manohar Lal and Uma S. Dubey
Department of Biological Sciences, Birla Institute of Technology of Science,
Pilani (BITS-PILANI), Pilani Campus, Rajasthan-333031, India

ABSTRACT

The objective of the present work was to purify bioactive components from camel milk and test them for antineoplastic properties. Camel milk was separated into casein and whey fractions and their cytotoxicity was assessed. Only camel milk whey and not casein were found to be cytotoxic to Hela cells. Further, camel milk whey was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), liquid chromatography-mass spectrometric (LC-MS) analysis and subsequent size exclusion chromatography. The cytotoxic potential of fractions obtained from chromatography were tested against the cervical cancer cell line, Hela. The present study indicated the presence of Lactoferrin (~77kDa) in the cytotoxic fractions purified from camel milk whey. This study indicates that camel milk whey and its components can be used as a nutraceutical with health benefis. It also suggests the potential of using camel milk lactoferrin as an important molecule with cyotoxic potential.

Key words: Camel milk, cervical cancer, chromatography, cytotoxicity, Hela, lactoferrin, LCMS, whey

Camel milk has therapeutic potential against many diseases including insulin-dependent diabetes mellitus (IDDM), infant diarrhea, hepatitis allergy, lactose intolerance, and alcohol-induced liver damage, and has been traditionally used for cancer prevention and treatment in Middle Eastern countries (Wernery, 2006). It has been traditionally believed that camel milk has both preventive and therapeutic potential against cancer. There is presence of numerous molecules with proven potential of antineoplastic activity, in other species (Dubey *et al,* 2016).

Studies have shown that camel milk works to combat and eradicate cancer hepatoma (HepG2) and human breast (MCF7) cancer cells, because camel milk contains high levels of lactoferrins, immunoglobulins, and iron-binding glycoprotein. Camel milk contains lactoperoxidase which possesses anti-tumour activity and peptidoglycan recognition protein which helps in fighting against cancer (Korashy *et al,* 2012). Lyophilised camel milk has been shown to inhibit the growth and proliferation of human breast cancer BT-474, laryngeal HE-p2 (Hasson *et al,* 2015). In addition, camel milk protein lactoferrin has been shown to inhibit the proliferation of human colorectal cancer HCT 116 cells by exerting antioxidant and DNA damage activities (Habib *et al,* 2013). Some of the *in vivo* studies have also shown the potent inhibitory effects of camel milk on pro-inflammatory, pro-angiogenic, and pro-fibrogenic cytokines (Alhaider *et al,* 2014).

Camel whey protein supplementation is shown to maintain a high concentration of cellular antioxidants and boost immune defenses that promote carcinogen detoxification (Ajarem *et al,* 2015). Camel whey proteins promote the production of IL-1β, IL-8, IL-6, and tumour necrosis factor (TNF-α) which enhances lymphocyte functions, chemotaxis, phagocytic activity, granulocyte, and NK cell activity (Gauthier *et al,* 2006; Rusu *et al,* 2010). Owing to these properties, camel whey protein supplementation is viewed as a non-pharmaceutical adjunct therapy in the treatment of cancer. In fact, camel whey protein is a new dietary supplementation to the management of free radicals and for the treatment of different health disorders (Badr *et al,* 2017).

The present study explores the cytotoxicity induced by the whey and casein fraction of camel milk. The composition of the bioactive whey fraction

SEND REPRINT REQUEST TO UMA S. DUBEY email: uma@pilani.bits-pilani.ac.in

is examined by LC-MS, and the impact of camel milk whey treatment on Hela cell migration is studied. Thereafter, we studied the cytotoxicity of camel milk whey fractions obtained by size exclusion chromatography, against Hela cells. The cytotoxic fractions were further subjected to SDS-PAGE analysis for the identification of protein components.

Materials and Methods

Milk processing

Camel milk samples were collected from healthy camels at NRCC, Jorbeer, Bikaner, Rajasthan, India. The experiments were conducted from 2017-19 at BITS Pilani, Pilani Campus. Fat components from the milk sample were removed by centrifugation at 8000 rpm for 30 minutes at 4°C to obtain skim milk. Whey was obtained from the skimmed milk by acid precipitation of caseins with 1 N HCl till pH 4.6 and centrifugation at 12000 rpm for 30 minutes at 4°C. The supernatant was saturated with ammonium sulphate (273 g/L of whey supernatant) by constant stirring at 800 rpm and incubated at 4°C overnight. It was subsequently spun at 10,000 rpm, 15°C for 15 minutes to obtain the whey pellet. Whey was extensively dialysed using dialysis membrane-60 (HiMedia) with PBS buffer (Saliha *et al,* 2013).

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

GE SimpliNano Spectrophotometer was used to quantitate the protein concentration of camel skim milk and camel milk whey was analysed by SDS PAGE on 12% gel using a Bio-Rad mini gel electrophoresis unit run. The proteins were diluted to 2 µg/µl with sample buffer and denatured for 10 min, at 100°C. Then the samples were added to each well, and electrophoresis was performed at 80 V. Proteins in the gel were stained by Coomassie Brilliant Blue R-250. The gel image was viewed using a gel documentation system (BioRad). Separated proteins were validated for identification using the stained wide range 6.5-200 kDa molecular weight marker (SIGMA #S8445) (Laemmli, 1970).

Measurement of cytotoxicity of fractions against Hela cells

The MTT assay determined the fractions efficacy to induce cytotoxicity against the Human cervical cancer cell line, HeLa (van Meerloo *et al,* 2011). These cells procured from NCCS, Pune, India were cultured at 37°C, 5% CO_2 in RPMI supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin mixture. Trypsin- EDTA solution was used for the detachment of cells. 5.0 x 10^4 cells/ well were incubated with fraction 1 to Fraction 10 in a 96-well cell culture plate under the conditions described above. After 24h and 48h, the media was aspirated. 20µl of MTT solution (5mg/ml in PBS) and 100 µL of media were added to each well. The plate was then re-incubated for 4 hours. To stop the reaction, the media were removed, and 150µL of DMSO was added to each well. The absorbance was measured at 630 nm using a Multiskan™ Thermo Scientific™ microplate reader. Cell viability was calculated as the percentage of the control wells using the following formula:

% Cell Viability = {O.D of treated cells / O. D of Control (without treatment)} X 100

Statistical analysis of the data was performed by GraphPad prism5 using one-way and two-way ANOVA followed by Bonferroni and Dunnett's multiple comparison test. Statistically significant differences with respect to control were represented by *P < 0.05, **P<0.001, ***P<0.0001, and NS = non-significant, respectively. Each experiment was done in triplicate and performed atleast thrice.

LC-MS Analysis of Proteins

The camel milk whey sample for its LC-MS analysis was outsourced to Vproteomics, New Delhi for identifying and determining the main features of proteins. The sample was reduced with 5 mM TCEP and then alkylated with 50mM iodoacetamide. This was followed by digestion with trypsin, and the digests were cleaned using a C18 silica cartridge to remove the salt and dried using a speed vac. The dried pellet was suspended in buffer A (5% acetonitrile, 0.1% formic acid). Peptide mixture analyses were performed using the EASY-nLC 1000 system (Thermo Fisher Scientific), coupled with Thermo Fisher-Q Exactive equipped with a nanoelectrospray ion source. RAW files generated were analysed with Proteome Discoverer (v2.2) against the Uniprot *Camelus* reference proteome database. This was used to identify and determine the proteins' main characteristic features in camel milk whey samples. Trypsin digestion of the sample was followed by nano-ESI LC-MS/MS analysis.

Size exclusion column chromatography

For size exclusion column chromatography, a column of diameter 1.5 cm and length 75 cm was used to separate camel milk whey proteins. Initially, 5g of Sephadex G 100 gel beads were soaked in phosphate buffer saline (pH 7.4) containing 0.02%

sodium azide then heated at 90°C for 5 hrs. Gel beads were degassed, loaded, and allowed to settle in the column. Then the column was washed with sodium phosphate buffer (pH 7.4), 17mg whey in a 5 ml buffer was loaded, 60 fractions were collected, and the absorbance was measured at 280 nm to quantitate the amount of protein content (Garcia Rojas *et al,* 2004). SDS PAGE was performed on 12% gel to profile protein present in fractions.

Silver staining of SDS PAGE gel

The gel was stained using silver stain kit (Himedia # ML123). Briefly, after running the gel, its fixation for 10 min, sensitisation for 1 min, and washing 3 times for 20 min was done. For staining, the gel was incubated in a silver staining solution for 20 min and rinsed for 1 min. A developing solution was added to the gel and incubated until the colour developed. In the end, a stopping solution was added, and the gel was washed before visualisation.

Results and Discussion

The protein profile of camel skim milk (CSM) obtained by SDS PAGE electrophoresis has been shown in lane 3 of Fig 1. It exemplifies the presence of all casein proteins and whey proteins. The camel milk whey (CMW) proteins are also depicted separately in lane 3. The major whey proteins are lactoferrin (Lf), camel serum albumin (CSA), multiple immunoglobulins (Ig), soluble TRAIL, and α-lactalbumin (α-LA) in CMW as well as CSM of these, lactoferrin and TRAIL are associated with an antineoplastic property. Additionally, CSM exemplifies the presence of the 4 casein proteins, namely, αs1-Casein (αs1-CN), αs2 - Casein (αs1-CN), beta Casein (β-CN), and kappa Casein (κ-casein), having molecular weight range of 23 kDa, 25 kDa, 24 kDa, 19 kDa, respectively. The molecular weight marker is depicted in lane 2.

MTT-based cell viability assay was performed against the HeLa cell line after 24h as well as after 48h treatment of Hela cells with whey and casein (Fig 2A and Fig 2B). A dose-dependent decline was observed in the viability of cells after whey treatment. The cytotoxicity after 48h was always more than after 24h, as to be expected. At 7.5 mg/mL concentration of whey, approximately 50% cell cytotoxicity was observed after 24h, indicating the IC50 of whey to be 7.5 mg/mL. Unlike whey, the casein treatment did not induce any cytotoxicity to the cells at the different concentrations after 24h and 48h as shown in Fig 2B. Therefore, only the whey fraction has been used for further studies.

In order to facilitate the identification of components present in whey fraction, it was subjected to analysis by LC-MS. The results of proteins having high protein scores (> 19) have been shown in table 1. The proteins corresponding to bands obtained upon fractionation have also been depicted in the table. It also facilitated the determination of the relative abundance of individual proteins. Numerous proteins such as alpha S1 casein, beta-casein, and lactoferrin could be identified. It also showed fragmented forms of kappa casein, milk fat globule EGF factor 8, α-lactalbumin, and alpha S1 casein. Amongst the molecules present, lactoferrin and α-lactalbumin are two molecules associated with anti-cancer properties, the latter only in combination with oleic acid. The SDS-PAGE gels also validate the presence of the proteins mentioned above.

Subsequently, gel filtration chromatography was conducted and the number of proteins present in fractions 1-60 has been depicted in Fig 3 which depicts the presence of proteins only in the initial fractions. Therefore, subsequently, further studies pertaining to SDS PAGE were conducted only on the initial 10 fractions.

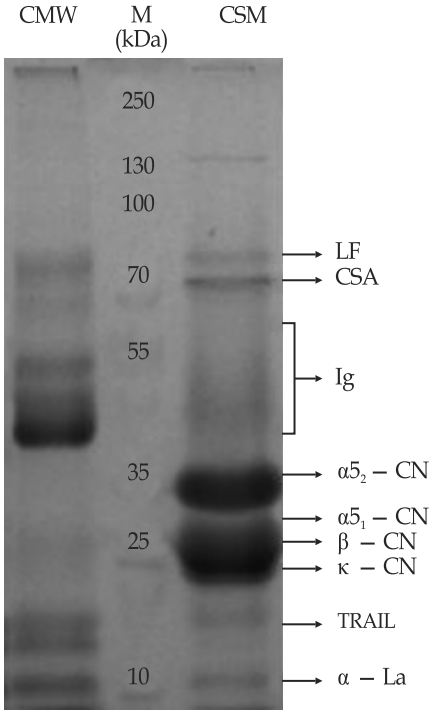

Fig 1. Protein profile of camel skim milk and camel milk whey by SDS-PAGE (L to R). Abbreviations: M (Molecular weight marker); CMW (Camel Milk Whey); CSM (Camel skimmed milk); CSA (Camel serum albumin); Ig (Immunoglobulin); αs1-Casein (αs1-CN); αs2 - Casein (αs1-CN); Beta Casein (β-CN); Kappa Casein (κ-casein); TRAIL (TNF-related apoptosis-inducing ligand), α-LA (α-Lactalbumin).

Fig 4 depicts the protein profile of the fractions obtained by gel filtration chromatography. The profile of molecular weight marker, whole milk (WM), whey (WH), and bovine lactoferrin (LF) are shown in the first 4 lanes sequentially. The other lanes were loaded with fractions 1-10. SDS- PAGE of only the fractions showing cytotoxicity (first 10) was performed. The functional significance of the proteins obtained has been shown in Table 2.

The fractions showed the presence of some biologically essential proteins, identified by their molecular weight. Lactoferrin and soluble TNF-related apoptosis-inducing ligand (TRAIL) are 2 proteins with antineoplastic properties were present out of which only the presence of lactoferrin could be confirmed by LCMS. Besides, these bands corresponding to camel serum albumin and heavy chain antibodies were also observed. The details of the fractions, along with the functions of proteins present, are shown in table 2.

The cytotoxic activity of these whey fractions was checked against HeLa cells to determine their potency against transformed cells. A decrease in per cent viability of the transformed cells was observed only in the initial 10 fractions and not the later fractions; therefore, keeping the objective in focus, further experiments were carried out using the initial 10 fractions only (data not shown).

Fig 5 depicts the cytotoxicity induced by whey fractions after a treatment of 24 hrs and 48 hrs. Fraction 1-3 did not induce any significant cytotoxicity after 24 hrs, although they could do so after 48 hrs. Fraction numbers 4-10 were able to induce statistically significant cytotoxicity at both time points. They further observed that more cytotoxicity is induced by all the fractions after 48 hrs compared to 24 hrs, as expected.

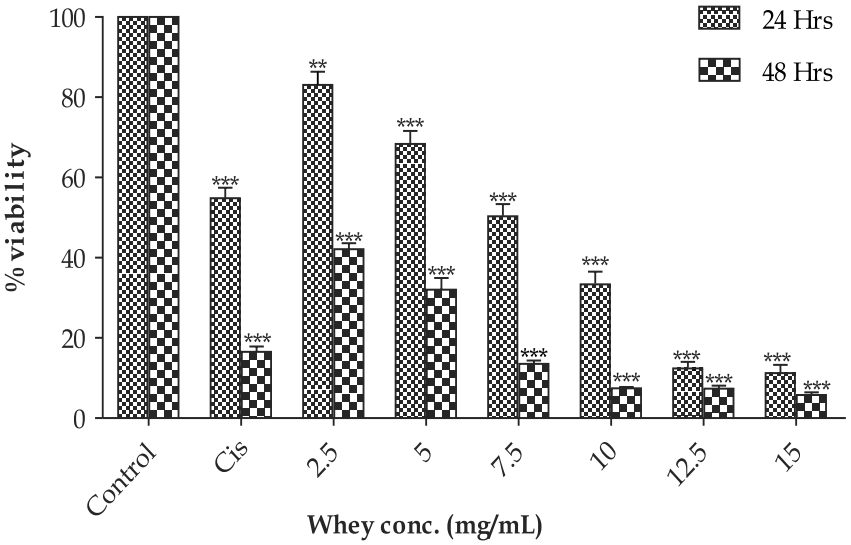

Fig 2A. Viability of HeLa cells after the treatment of camel milk whey by MTT assay after 24 and 48 hrs. The untreated cells (negative control) and cisplatin IC50 (14.9 μM) treated cells (positive control) are also depicted.

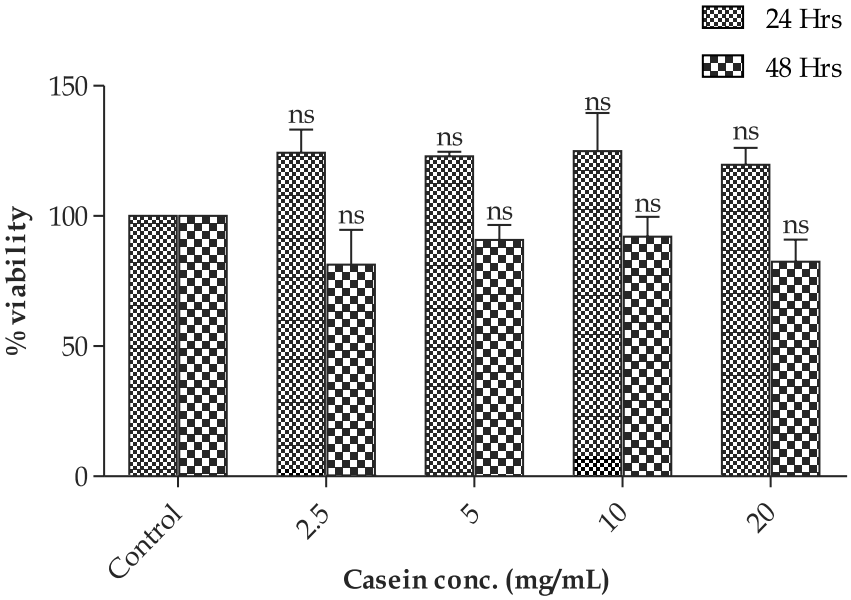

Fig 2B. Viability of HeLa cells after the treatment of camel milk casein by MTT assay after 24 and 48 hrs. The untreated cells are used as a control.

This study suggested that the cytotoxicity of camel milk is largely associated with its whey fraction but not its casein fraction. The whey fractions were purified by size exclusion chromatography and further subjected to SDS-PAGE analysis for the identification of proteins. Proteins with molecular weights 20kDa and 77kDa were found in the cytotoxic fractions purified by size exclusion chromatography. Literature suggests the possibility of soluble TNF-related apoptosis-inducing ligands (TRAIL) of molecular weight 20kDa and Lactoferrin of molecular weight 77kDa as the cytotoxic molecules in human

Table 1. Analysis of camel (*Camelus dromedarius*) milk whey protein by LC-MS.

Accession	Description	Score	Coverage	Peptide spectrum match	Amino Acid	Mol. wt [kDa]	Calc. pI
W0K8B9	κ-casein	118.52	60.13	45	153	17.1	8.60
K7DXB9	α-s1-casein	71.97	40.99	33	222	25.8	5.08
F5BZ34	Milk fat globule EGF factor 8	67.39	37.07	30	294	32.8	8.03
M1E4K4	β-casein	43.36	39.22	18	232	26.2	5.58
W6GH05	Lactoferrin	28.67	12.01	13	708	77.3	8.24
A0A2H4WWA5	A-lactalbumin	24.36	42.31	12	52	6.1	4.81
A0A0U2KTX5	Single variable on heavy chain (VHH)	1.73	7.38	1	122	13.0	10.8

Table 2. Protein profile of fractions.

Protein	Molecular Weight	Function	Fraction number
Lactoferrin	77	Immunomodulatory, antimicrobial and anticancer	7, 8, 9 & 10
Camel serum albumin	68	Transport of biomolecules	6, 7 & 8
Heavy chain antibody	50	Immune response	7, 8 & 9
TRAIL	20	Anticancer property	4, 6 & 7

milk (Davanzo, 2013). Besides these, the presence of Ig heavy chain and camel serum albumin was also obtained in the fractions, but these are not associated with any anticancer activity. Camel milk casein, although not cytotoxic against transformed cells but has been used as a carrier for natural and synthetic drugs (Mittal *et al*, 2021). Camel milk whey was found to be cytotoxic to these cells at 24 and 48 hrs in a dose-dependent manner. This indicates that the cytotoxicity of camel milk is primarily associated with the whey fraction. Besides whole milk, some scientists have also studied the anti-cancer property of camel whey protein hydrolysates. Animal models for colon and mammary tumourigenesis have shown that whey proteins are better than other dietary proteins in suppressing tumor development (Parodi, 2007). These studies indicate that camel milk whey can be potentially utilised as a functional ingredient in nutraceuticals. In our study camel milk casein was not toxic to cervical cancer cell line Hela.

LC-MS was used to identify the other proteins in the whey fraction. Although, it showed the presence of many proteins like lactoferrin, α-lactalbumin, fragmented forms of kappa casein, milk fat globule EGF factor 8, and alpha S1 casein. Amongst the molecules present, lactoferrin is the only molecule associated with anti-cancer properties. Although the molecule of soluble TRAIL could be identified by SDS PAGE the same could not be verified by LCMS. One of the possible reasons for missing TRAIL in LCMS could be the nonavailability of camel milk TRAIL sequence in the database used for identifying proteins. We know that camel milk TRAIL has not been studied sufficiently.

It is well known that camel milk contains numerous bioactive molecules with immunomodulatory, antimicrobial, and anticancer abilities. Camel milk proteins are highly thermostable besides being resistant to acid hydrolysis. The SDS-

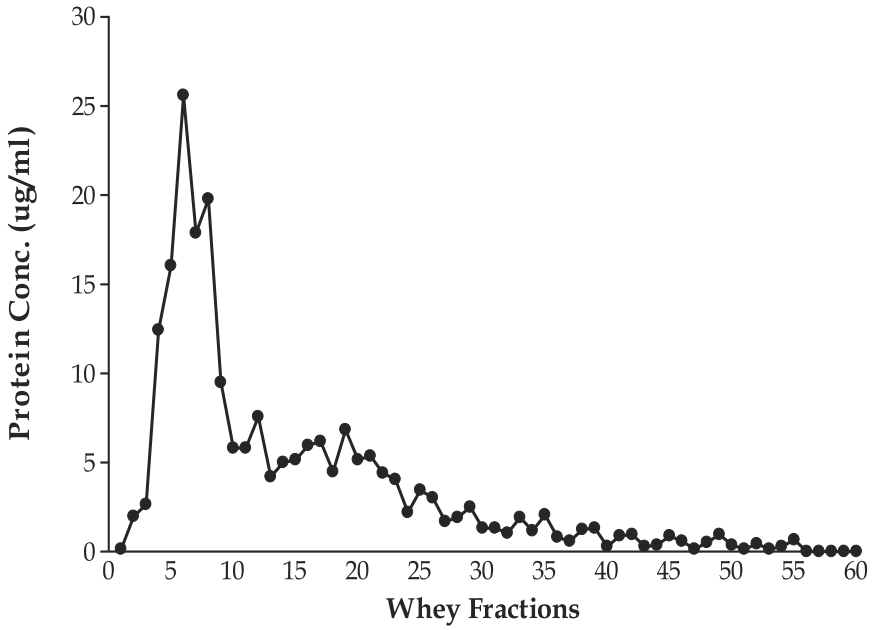

Fig 3. The protein content of fractions separated by Gel Filtration Chromatography.

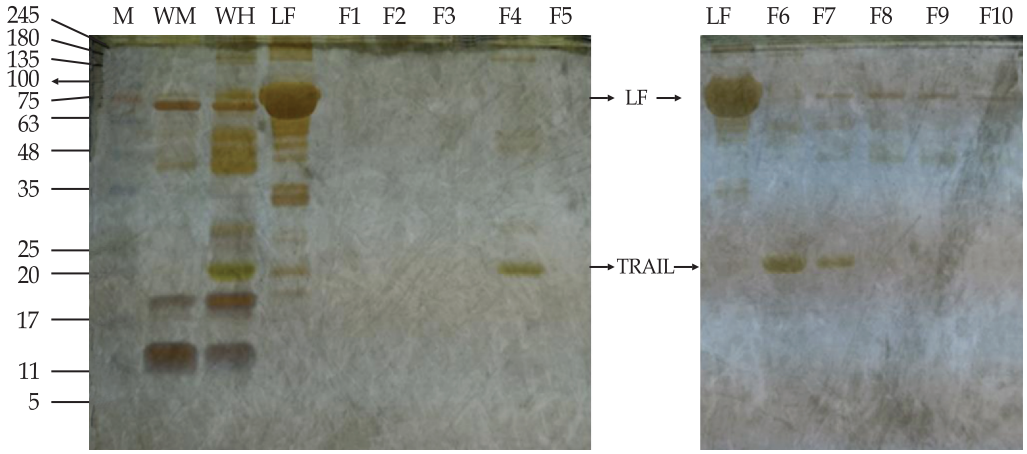

Fig 4. Protein profile of fractions obtained from whey using gel filtration column chromatography by SDS-PAGE (L to R). Abbreviations: M (Molecular weight marker); WM (Whole Milk); WH (Whey), LF (Bovine Lactoferrin) F1 – F10 (Fraction 1 to Fraction 10).

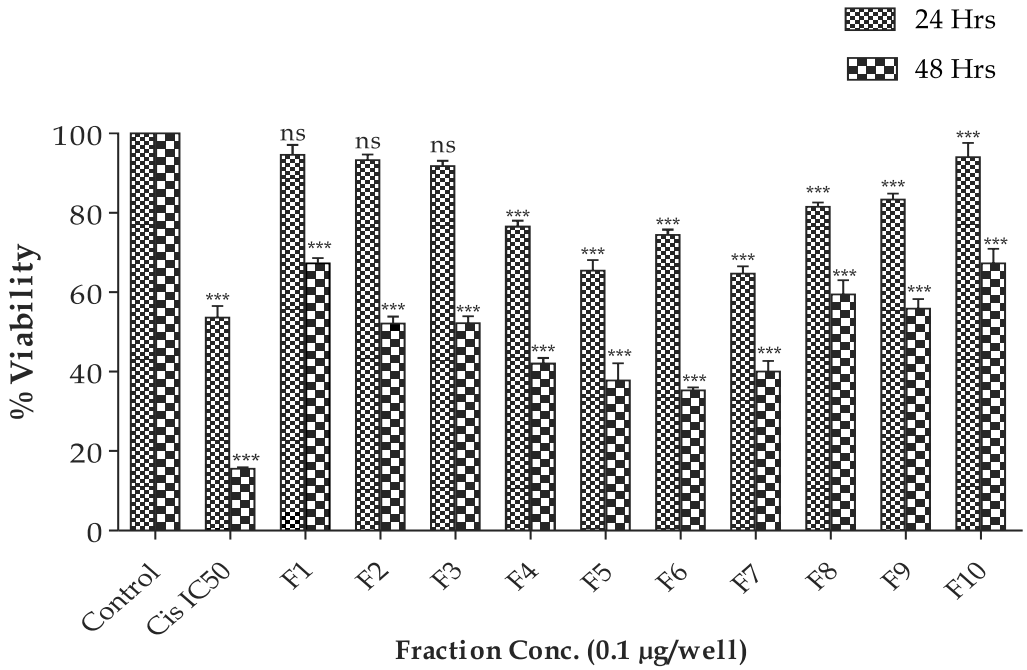

Fig 5. Viability of HeLa cells after the treatment of 0.1 µg/well of indicated protein fraction by MTT assay after 24 and 48 hrs. The untreated cells (negative control) and cisplatin IC50 (14.9 µM) treated cells (positive control) are also depicted.

PAGE analysis of this study of skimmed camel milk and whey showed additional proteins such as different caseins, serum albumin, multiple immunoglobulins, and α-lactalbumin, respectively (Lal *et al,* 2020; Mittal *et al,* 2021).

Camel milk's whey significant proteins contain the entire array of normal antibodies besides having the unique featured camelid antibodies, single-domain antibodies (SdAb) containing only the heavy chain and not the light chain; they retain their specificity and stability i.e., the only heavy chain immunoglobulins antibodies (VHH antibodies) (Desmyter *et al,* 2001). They are often referred to as nanobodies due to their small size. Therefore, the unique features of these antibodies and the presence of other biologically important molecules and their derivatives confer camel milk with unique medicinal properties (Abdel Gader and Alhaider, 2016).

Few authors have recently studied the ability of mammalian milk and its fractions to kill cancers (Shariatikia *et al,* 2017). They have studied the effect of milk, casein, and whey proteins derived from many different mammals on MCF7. Their results showed that mare, donkey, cow, and camel milk, casein,

and whey proteins have potent cytotoxic activity against MCF7 cells in a dose-dependent manner. In contrast, sheep and goat milk, whey and casien did not reveal any cytotoxic activity. Korashy *et al* (2012) have reported that camel milk has the ability to significantly inhibit the induction of the Cyp1A1, a cancer-activating gene. Further, it also induces NQO1, a cancer chemo-preventive gene in murine hepatoma Hepa 1c1c7 cells. Both these functions were studied at the transcriptional and post-transcriptional levels. It was observed that the survival of HepG2 cells was significantly reduced upon incubation with camel milk. Further, they observed that camel milk significantly induced caspase-3 and DR4 mRNA expression levels. The induction of Caspase-3 was blocked by the action of Act-D. This indicates that camel milk increased the caspase-3 mRNA level by the de novo synthesis of RNA. It was further observed that pretreatment of cells with MAPK inhibitors alone, slightly, but not significantly decreased the basal expression level of caspase-3 mRNA. Furthermore, it was reported that the induction of caspase-3 mRNA by camel milk in HepG2 cells was significantly decreased by both the JNK and p38 MAPKs inhibitors and was potentiated by an ERK inhibitor (Korashy *et al,* 2012).

Our study also exemplifies the ability of camel milk whey to be cytotoxic to the cervical cancer cell line, HeLa. Earlier, we also purified the whey proteins to get lactoferrin by a novel pH-dependent procedure (Dubey *et al,* 2022). In this work, SDS-PAGE and LC-MS analysis of the lactoferrin fraction was done for its identification and characterisation. Furthermore to evaluate of the bioactivity of the isolated lactoferrin MTT-based cytotoxicity against the Hela cells was also conducted (Mahala *et al,* 2022).

Similarly, numerous studies point towards the protective character of lactoferrin against malignancies. Bovine lactoferrin has been reported to inhibit the development of experimental metastases in mice with chemically induced tumours (Iigo *et al,* 2009). Bovine lactoferrin-mediated inhibition of tumour growth might be related to apoptosis of these cells, induced by the activation of the Fas signaling pathway (Fujita *et al,* 2004). Treatment of lactoferrin knockout mice with lactoferrin post-chemotherapy accelerated the reconstitution of the immune system, reducing the chances for infection following chemotherapy treatment (Ye *et al,* 2014).

Lactoferrin can scavenge free iron in fluids and inflammed and infected sites, suppressing free radical-mediated damage and decreasing the availability of the metal to pathogens and cancer cells. Also, lactoferrin hinders migration in a model of human glioblastoma by reverting an epithelial-to-mesenchymal transition like process (Adlerova *et al,* 2008; Cutone *et al,* 2020).

Furthermore, camel milk lactoferrin reduces the proliferation of colorectal cancer cells and exerts antioxidant and DNA damage inhibitory activities (Habib *et al,* 2013). The administration of bovine Lactoferrin in a randomised placebo-controlled clinical trial setting has also been reported to elicit beneficial effects for blocking the growth of polyps that are often thought to lead to colon cancer (Kozu *et al,* 2013). Additionally, lactoferrin knockout mice demonstrated a significant susceptibility to inflammation-induced colorectal dysplasia. Lactoferrin carries out different functions owing to its ability to activate specific signaling pathways. It is therefore, of utmost importance to consider the iron saturation rate when carrying out *in vitro* and *in vivo* experiments (Cutone *et al,* 2020).

Contrary to the notion of effectiveness of oral administration of camel lactoferrin, many other therapeutic proteins typically require other invasive routes of administration (Leader *et al,* 2008). Oral administration of bovine Lactoferrin inhibits carcinogenesis in the colon and other organs in rats and lung metastasis in mice. A likely mechanism by which bovine lactoferrin mediates its anti-carcinogenesis effects is by enhanced expression of cytokines and subsequent activation of immune cells (Iigo *et al,* 2009). Although the level of lactoferrin is comparable to cow's milk, even then its bioactivity is slightly higher (Conesa *et al,* 2008; Narmuratova *et al,* 2006).

Studies have shown high levels of TRAIL in human milk and colostrum. Its presence has been implicated as one of the many molecules for anticancer properties in human milk, but camel milk soluble TRAIL has not been studied sufficiently. TRAIL is a cytokine that is produced and secreted by most normal tissue cells. It causes apoptosis primarily in tumour cells by binding to specific death receptors. TRAIL and its receptors have been used as the targets of several anticancer therapeutic modalities. TNF family members, the ligand TRAIL, is primarily expressed as a type 2 transmembrane protein, which proteases can process to release the soluble form (Shepard and Badley, 2009). The protein profile of whole camel milk resulting from commercial thermal treatment was examined by liquid Chromatography combined with Tandem Mass Spectrometry (LC-

MS/MS) to identify proteins. In this study, total 807 proteins were identified which were mainly involved in biological processes like metabolic process and cellular processes and mostly related to signaling pathways including RNA transport, PPAR signaling pathway, and ribosome (Li *et al,* 2020).

In our study, camel milk whey has been fractionated by size exclusion chromatography to yield proteins with potential cytotoxicity toward Hela cells. The present fraction study indicated lactoferrin to be the most vital anticarcinogenic molecule in camel milk whey. sTRAIL, the other potential molecule, needs to be further studied.

Acknowledgement

This work was supported by the Department of Science and Technology (DST) Rajasthan, India under (Sanction No.P.7 (3) vipro/R&D/2016 5945-56 dated 10/8/16) and professional development fund by Birla Institute of Technology and Science, Pilani (BITS Pilani), Pilani Campus, Rajasthan, India. Neelam Mahala acknowledges the Department of Science and Technology (DST), India for the Innovation in Science Pursuit for Inspired Research [INSPIRE] fellowship [DST/INSPIRE Fellowship/2016/IF160137], and Aastha Mittal acknowledges DST Rajasthan for financial support.

References

Abdel Gader AGM and Alhaider AA. The unique medicinal properties of camel products: A review of the scientific evidence. Journal of Taibah University Medical Sciences. 2016; 11:98-103.

Adlerova L, Bartoskova A and Faldyna M. Lactoferrin: A review. Veterinarni Medicina. 2008; 9:457-468.

Ajarem J, Allam AA, Ebaid H, Maodaa SN, Al-Sobeai SM, Rady AM and Sabri MI. Neurochemical, structural, and neurobehavioural evidence of neuronal protection by whey proteins in diabetic albino mice. Behavioural and Brain Functions. 2015; 11(1):1-8.

Alhaider, AA, Abdel Gader AGM, Almeshaal N and Saraswati S. Camel milk inhibits inflammatory angiogenesis *via* downregulation of proangiogenic and proinflammatory cytokines in mice. Acta Pathologica Microbiologica Et Immunonologica Scandinavica. 2014; 122(7):599-607.

Badr G, Ramadan NK, Sayed LH, Badr BM, Omar HM and Selamoglu Z. Why whey? Camel whey protein as a new dietary approach to the management of free radicals and for the treatment of different health disorders. Iranian Journal of Basic Medical Sciences. 2017; 20(4): 338.

Conesa C, Sánchez L, Rota C, Pérez MD, Calvo M, Farnaud S and Evans RW. Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2008; 150(1):131-139.

Cutone A, Colella B, Pagliaro A, Rosa L, Lepanto MS, Bonaccorsi di Patti MC, Valenti P, Di Bartolomeo S and Musci G. Native and iron-saturated bovine Lactoferrin differently hinder migration in a model of human glioblastoma by reverting epithelial-to-mesenchymal transition-like process and inhibiting interleukin-6/STAT3 axis. Cell Signalling. 2020; 65:109461.

Davanzo R, Zauli G, Monasta L, Vecchi Brumatti L, Abate MV, Ventura G, Rimondi, E, Secchiero P and Demarini S. Human colostrum and breast milk contain high levels of TNF-related apoptosis-inducing ligand (TRAIL). Journal of Human Lactation. 2013; 29:23-25.

Desmyter A, Decanniere K, Muyldermans S and Wyns L. Antigen specificity and high affinity binding provided by one single loop of a camel single-domain antibody. Journal of Biological Chemistry. 2001; 276:26285-26290.

Dubey US, Mahala N, Mittal A and Lal M. Methods for Isolation of Lactoferrin from Camel Milk. India Patent Application No. 202211003167. 2022.

Dubey US, Lal M, Mittal A and Kapur S. Therapeutic potential of camel milk. Emirates Journal of Food and Agriculture. 2016; 28:164-176.

Fujita K, Matsuda E, Sekine K, Iigo M and Tsuda H. Lactoferrin enhances Fas expression and apoptosis in the colon mucosa of azoxymethane-treated rats. Carcinogenesis. 2004; 25:1961-1966.

Habib HM, Ibrahim WH, Schneider-Stock R and Hassan HM. Camel milk lactoferrin reduces the proliferation of colorectal cancer cells and exerts antioxidant and DNA damage inhibitory activities. Food Chemistry. 2013; 141:148-152.

Hasson SS, Al-Busaidi, JZ, Al-Qarni ZA, Rajapakse S, Al-Bahlani S, Idris MA and Sallam TA. *In vitro* apoptosis triggering in the BT-474 human breast cancer cell line by lyophilised camel's milk. Asian Pacific Journal of Cancer Prevention. 2015; 16(15):6651-6661.

Iigo M, Alexander DB, Long N, Xu J, Fukamachi K, Futakuchi M, Takase M and Tsuda H. Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine. Biochimie. 2009; 91:86-101.

Gauthier SF, Pouliot Y and Saint-Sauveur D. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. International Dairy Journal. 2006; 16(11):1315-1323.

Garcia Rojas, EE, dos Reis Coimbra JS, Minim LA, Zuniga ADG, Saraiva SH and Rodrigues Minim VP. Size-exclusion chromatography applied to the purification of whey proteins from the polymeric and saline phases of aqueous two-phase systems. Process Biochemistry. 2004; 39:1751-1759.

Kamal H, Jafar S, Mudgil P, Murali C, Amin A and Maqsood S. Inhibitory properties of camel whey protein hydrolysates toward liver cancer cells, dipeptidyl peptidase-IV, and inflammation. Journal of Dairy Science. 2018; 101(10):8711-8720.

Korashy HM, EI Gendy MAM, Alhaider AA and EI-Kadi AO. Camel milk modulates the expression of aryl

hydrocarbon receptor-regulated genes, Cyp1a1, Nqo1, and Gsta1, in murine hepatoma Hepa 1c1c7 cells. Journal of Biomedicine and Biotechnology 782642. 2012.

Kozu T, Iinuma G, Ohashi Y, Saito Y, Akasu T, Saito D, Alexander DB, Iigo M, Kakizoe T and Tsuda H. Effect of orally administered bovine lactoferrin on the growth of adenomatous colorectal polyps in a randomised, placebo-controlled clinical trial. Cancer Prevention Research. 2009; 2(11):975-983.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lal M, Saumya KU, Mahala N, Runthala A and Dubey US. Camel milk α-lactalbumin as a potential anticancer molecule: a bioinformatics analysis. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2020; 11:38-52.

Leader B, Baca QJ and Golan DE. Protein therapeutics: a summary and pharmacological classification. Nature Reviews Drug Discovery. 2008; 7:21-39.

Li RR, Yue HT, Shi ZY, Shen T, Yao HB, Zhang JW, Gao Y and Yang J. Protein profile of whole camel milk resulting from commercial thermal treatment. LWT. 2020; 134:110256.

Mahala N, Mittal A, Lal M and Dubey US. Isolation and characterisation of bioactive lactoferrin from camel milk by novel pH-dependent method for large scale production. Biotechnology Reports. 2022; 36:e00765.

Mahala N, Mittal A and Dubey US. Medicinal Potential of Camel Milk Lactoferrin. In (Ed.), Dairy Processing - From Basics to Advances [Working Title]. IntechOpen. 108316. 2022.

Mittal A, Mahala N, Krishna KV, Dubey US and Dubey SK. Calcium chloride linked camel milk derived casein nanoparticles for the delivery of sorafenib in hepatocellular cells. Biocell. 2021; 46:127-136.

Narmuratova M, Konuspayeva G, Loiseau G, Serikbaeva A, Barouh N, Montet D and Faye B. 2006. Journal of Camel Practice and Research. 2006; 13(1):45-50.

Parodi PW. A Role for Milk Proteins and their Peptides in Cancer Prevention. Current Pharmaceutical Design. 2007; 13(8):813-828.

Rusu D, Drouin R, Pouliot Y, Gauthier S and Poubelle PE. A bovine whey protein extract stimulates human neutrophils to generate bioactive IL-1Ra through a NF-κB-and MAPK-dependent mechanism. The Journal of Nutrition. 2010; 140(2):382-391.

Saliha, SAZ, Dalila A, Chahra S, Saliha BH and Abderrahmane M. Separation and characterisation of major milk proteins from Algerian Dromedary (*Camelus dromedarius*). Emirates Journal of Food and Agriculture. 2013; 25:283-290.

Shariatikia M, Behbahani M and Mohabatkar H. Anticancer activity of cow, sheep, goat, mare, donkey and camel milks and their caseins and whey proteins and in silico comparison of the caseins. Molecular Biology Research Communications. 2017; 6:57-64.

Shepard BD and Badley AD. The biology of TRAIL and the role of TRAIL-based therapeutics in infectious diseases. Anti-Infective Agents in Medicinal Chemistry. 2009; 8:87-101.

Van Meerloo J, Kaspers GJL and Cloos J. Cell sensitivity assays: the MTT assay. Methods in Molecular Biology. 2011; 731:237-245.

Wernery U. Camel milk, the white gold of the desert. Journal of Camel Practice and Research. 2006; 13:15.

Ye Q, Zhen Y, Fan S, Qin Z, Li N, Tang A and Huang J. Lactoferrin deficiency promotes colitis-associated colorectal dysplasia in mice. PloS one. 2014; 9:103298.