



## Research Article

# Isolation and characterization of bioactive lactoferrin from camel milk by novel pH-dependent method for large scale production

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## ABSTRACT

The present article exemplifies a novel method to isolate highly purified bioactive lactoferrin from camel milk. Cytotoxicity of lactoferrin against the Hela cells was used to evaluate its bioactivity. SDS-PAGE and LC-MS analysis was done for its identification and characterization. The purified camel milk lactoferrin was found to be 708 amino acids in length with a molecular weight of 77.3 kDa and a pI value of 8.24. This pH-dependent isolation procedure ensures the retention of bioactive lactoferrin from camel milk. The importance of the present work lies in its simplicity and scalability for manufacturing bioactive lactoferrin at an industrial level.

## 1. Introduction

Milk is a vital source of nutrition with powerful immunomodulatory properties [1]. It has been known as nature's most essential and complete food for over 6 billion people worldwide [2]. The multifunctional roles mediated by bioactive compounds present in the milk include their ability to act as antimicrobial, anti-inflammatory, anti-oxidative, anti-cytotoxic, anticancer, immunomodulatory, and transporting molecules [3]. Neonates are protected from various microbial infections and cancer, but their good health is dependent on the presence of bioactive compounds in the colostrum and the milk [4]. Numerous studies have been conducted on milk from different animals. Recently, the therapeutic potential of Dromedary camel milk (*Camelus dromedarius*) milk has received attention worldwide.

Camel milk has unique physical, chemical, and biochemical nutritional qualities and has been traditionally consumed in middle eastern countries for its health benefits and medicinal properties [5, 6]. It also has known therapeutic and prophylactic benefits against cancer. Camel milk contains numerous immunoglobulins, alpha-lactalbumin, lactoperoxidase, casein, lysozyme, amylase, and lactoferrin [7]. It has been noted that despite the lack of refrigeration, camel's milk remains unspoiled for several days; this may be due to the antimicrobial activity of specific proteins, such as lactoferrin, contained in camel's milk [8]. The property of high thermo- stability and acid pH hydrolysis resistance

associated with camel milk proteins is an additional advantage [7]. Furthermore, camel milk-derived nanoparticles are being explored for their role in drug delivery [37].

Lactoferrin is an iron-binding glycoprotein belonging to the transferrin family. It possesses various anti-microbial and cancer-fighting properties [9,11]. Lactoferrin obtained from bovine and human milk has been used in different products, such as infant formulas, probiotics, supplemental tablets, cosmetics, and natural solubilizers of iron in food [10–12].

Multiple conventional purification methods like separation using cryogel column [13], semi-batch foaming process [14], and ultrafiltration coupled with cation exchange membranes [15] as well as non-conventional two-phase aqueous extraction and reverse micellar extraction [16] have been used for purifying lactoferrin from milk. The major disadvantages of these methods include loss of activity, less yield, cumbersome procedure, economic burden, and environmental impact. The present study aims to develop a simple, novel pH-dependent method to isolate lactoferrin from camel milk in ample amounts. This procedure enables better purification and large-scale production of lactoferrin for industrial and commercial purposes.

Abbreviations: OD, Optical density;  $\alpha$ S2-CN,  $\alpha$ S2-Casein; rpm, Revolutions per minute.

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**Abbreviations**

|                  |   |
|------------------|---|
| $\mu\text{L}$    | microliter  |
| HCl              | Hydrochloric acid   |
| NaOH             | Sodium hydroxide  |
| SM               | Skim Milk   |
| SDS-PAGE         | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis        |
| bLf              | Bovine lactoferrin  |
| LC-MS/MS         | Liquid Chromatography-Mass Spectroscopy                           |
| TCEP             | tris(2-carboxyethyl) phosphine                                    |
| IAM              | Iodoacetamide   |
| STR              | Short Tandem Repeat   |
| NCCS             | National center for cell science                                  |
| MEM              | Minimal essential medium  |
| FBS              | Fetal bovine serum  |
| $\text{NaHCO}_3$ | Sodium Bicarbonate  |
| EDTA             | Ethylenediaminetetraacetic acid                                   |
| MTT              | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt |
| DMSO             | Dimethyl sulfoxide  |
| SEM              | Standard error of means   |

|                        |  |
|------------------------|--|
| ANOVA                  | Analysis of variance   |
| CSA                    | Camel serum albumin  |
| Ig                     | Immunoglobulin   |
| $\alpha\text{s1-CN}$   | $\alpha\text{s1-Casein}$   |
| $\beta\text{-CN}$      | Beta Casein  |
| $\kappa\text{-casein}$ | Kappa Casein   |
| TRAIL                  | TNF-related apoptosis-inducing ligand                              |
| $\alpha\text{-LA}$     | $\alpha\text{-Lactalbumin}$  |
| w/v                    | weight/volume  |
| N                      | Normal   |
| PSM                    | Peptide spectrum match   |
| AA                     | Amino acid   |
| MW                     | Molecular weight   |
| kDa                    | kilodalton   |
| OS                     | Organism Name  |
| OX                     | Organism Identifier  |
| GN                     | Gene Name  |
| PE                     | Protein Existence  |
| SV                     | Sequence Version   |
| DST                    | Department of Science and Technology                               |
| INSPIRE                | India for the Innovation in Science Pursuit for Inspired Research. |

**2. Materials and methods****2.1. Camel milk sample collection**

Camel milk samples were aseptically collected from healthy camels by experts from Sarika Raika Milk Bhandar, Jaipur, Rajasthan, India. Sodium azide (0.02% w/v) was added to the skim milk to prevent bacterial growth. It was immediately transported on ice and stored at  $-20^\circ\text{C}$  for further use.

**2.2. Separation of whey from camel milk**

The milk sample was centrifuged for 5000 rpm for 30 min at  $4^\circ\text{C}$  using a super speed centrifuge (Sorvall™ LYNX 4000, Thermo Fisher Scientific India Pvt Ltd, India) to remove the uppermost creamy layer that contains fat. The defatted skimmed milk obtained was filtered using Whatman Qualitative Filter Paper (Grade-1) to remove cream traces, if any [17]. The defatted milk was diluted with an equal volume of milli Q water. The initial pH of the sample was recorded using a pH meter (Mettler-Toledo Benchtop pH meter, Mettler-Toledo India Private Limited, India), then 1 N Hydrochloric acid (HCl) was added slowly with constant stirring using a magnetic stirrer (Thermo Scientific™ RT Magnetic Stirrer, Thermo Fisher Scientific India Pvt Ltd, India) to sample until pH reached 4.6 to precipitate casein [18]. This was followed by centrifugation at 12,000 rpm for 30 min at  $4^\circ\text{C}$  to remove the casein pellet, as illustrated in Fig. 1. [19]. The whey supernatant obtained was stored in a refrigerator at  $-80^\circ\text{C}$  for further processing and isolating lactoferrin.

**2.3. Isolation of lactoferrin from camel milk whey**

The whey sample separated above was used to isolate lactoferrin, as detailed in the flowchart (Fig. 1) and described ahead. To the whey supernatant, 2 N Sodium hydroxide (NaOH) was added slowly until pH 6.8 was reached. An equal volume of 45% ammonium sulfate solution was added while stirring at 100 rpm. The speed was gradually increased to 420 rpm and continued for 1 hour at room temperature. The precipitated protein was removed by centrifugation at 10,000 rpm for 30 min at  $4^\circ\text{C}$ . 1 N HCl was slowly added (with constant stirring) to the supernatant till pH 4.0 was reached. This was followed by adding 2 N

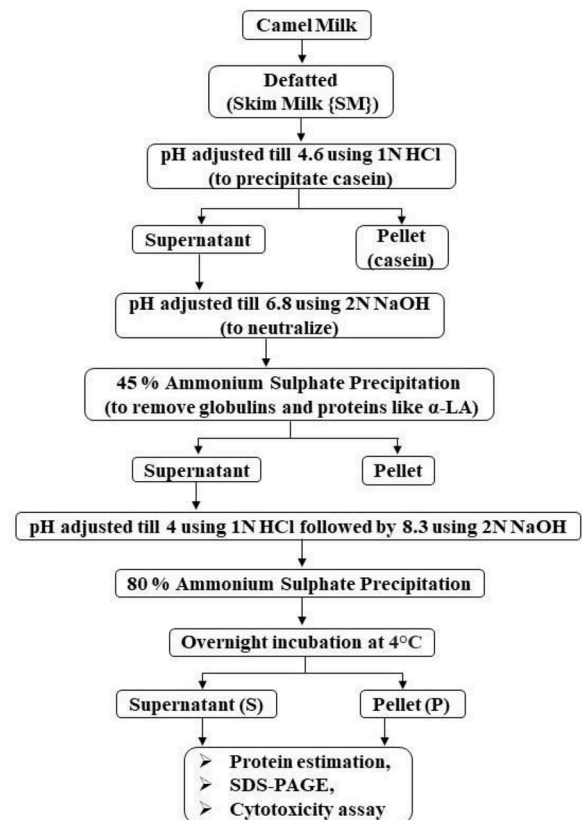


Fig. 1. Isolation of Lactoferrin from camel milk.

NaOH slowly till the pH became 8.3. The pH was adjusted to pH 4 to precipitate the caseins from the camel milk sample thereby increasing the purity of our protein of interest. It was adjusted to pH 8.3 by adding 2 N NaOH to precipitate lactoferrin, as 8.3 is the pI of camel milk lactoferrin. At pH 8.3, an equal volume of 80% ammonium sulfate solution was added (with constant magnetic stirring at 100 rpm) and the rate

gradually increased to 420 rpm and further stirred for 1 hour. The mixture was incubated overnight at 4 °C (while being stirred) to precipitate lactoferrin. Finally, centrifugation at 4000 rpm for 10 min at 4 °C was used to harvest all the lactoferrin. This isolated lactoferrin precipitate was then dissolved in water and dialyzed thrice using dialysis membrane 60 (HiMedia) with milli Q water and finally against 10 mM sodium phosphate buffer (pH 7.4). After Dialysis, the clear solution was stored at –20 °C and used for further studies. An Indian patent has been filed for this procedure [20].

#### 2.4. Protein profiling by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

GE SimpliNano Spectrophotometer was used to quantitate the protein concentration. The samples obtained at each step were subjected to SDS-PAGE according to the procedure developed by Laemmli [21]. Briefly, the samples were denatured in 2X sample buffer containing 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue, and boiled for 5 min., Samples (15 µL) were loaded each well in the gel (12% running gel and 4% stacking gel). The gel was run on a Mini Protean® Tetra-Cell instrument (BioRad) at a constant voltage of 80 kV. The protein bands on the gel were stained by Coomassie Brilliant Blue R-250 for 1 hour and destained in methanol (15%): acetic acid (10%): water (75%) solution overnight. The gel was observed using a gel documentation system (BioRad Gel DocTM XR imaging system). Separated proteins were validated for identification using the molecular weight marker (6.5–200 kDa) Bovine lactoferrin (bLf) was used as a positive control to identify lactoferrin by SDS-PAGE.

#### 2.5. Liquid chromatography-mass spectroscopy

The isolate purified earlier by the pH-dependent procedure was used for Liquid Chromatography-Mass Spectroscopy (LC-MS/MS) analysis to confirm the presence of lactoferrin and to further characterize it.

#### 2.6. Peptide mixtures preparation

The lactoferrin-containing fraction was reduced with 5 Mm TCEP ((tris(2-carboxyethyl) phosphine) and further alkylated with 50 mM IAM (iodoacetamide) and subsequently digested with Trypsin/lysate ratio) for 16 h at 37 °C. Digests were cleaned with a C18 silica cartridge to remove the salt and dried using a speed vacuum drier. The dried pellet was suspended in buffer A (5% acetonitrile, 0.1% formic acid).

#### 2.7. Protein hydrolysate analysis

The analysis was performed using EASY-nLC 1000 system (Thermo Fisher Scientific) coupled to Qexactive (Thermo Fischer Scientific) equipped with a nano electrospray ion source. The generated mass spectrometric data were analyzed with Proteome Discoverer (v2.2) against the Uniprot *Camelus* reference proteome database. We thank VProteomics, New Delhi for performing the LC-MS/MS on our camel milk lactoferrin isolate.

#### 2.8. Culture of HeLa cells

Short Tandem Repeat (STR) profiled certified human cervical (HeLa) cell line was obtained from the National center for cell science (NCCS), Pune, India. It was cultured at 37 °C, 5% CO<sub>2</sub> in a Minimal essential medium (MEM; Gibco, Thermo Fisher Scientific), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific), Sodium Bicarbonate (NaHCO<sub>3</sub>; Himedia India, Ltd. Mumbai, India), and 1% antibiotic solution containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Thermo Fisher Scientific). Before treatment, cells were grown to 60–70% confluence in a tissue culture flask (Tarson). Adhered cells were detached with 1X Trypsin EDTA

solution containing 0.05% Trypsin (Gibco, Thermo Fisher Scientific) solution rinsed in phosphate-buffered saline and transferred into a fresh medium.

#### 2.9. Cytotoxicity of camel milk lactoferrin

The cytotoxicity of camel lactoferrin-treated against HeLa cervical cancer cells was performed by the MTT assay. The efficacy of the camel milk lactoferrin isolate to alter HeLa cells proliferation was determined by measuring the capacity of reducing enzymes present in viable cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) Sigma-Aldrich Chemicals Private Limited, India) to its formazan crystals. HeLa cells were seeded into a 96-well plate at the concentration of  $4 \times 10^3$  cells/well in 100 µl of the medium. After 24 h at 37 °C, these cells were treated with different concentrations i.e., 25, 50, 75, 100, 125, 150, 175 and 200 µg/mL of camel milk lactoferrin isolate for 24 h and 48 h, respectively. Cisplatin (Merk, Sigma-Aldrich Chemicals Private Limited, India) treated cells and untreated cells were used as the positive and negative controls, respectively. The supernatant was aspirated after appropriate incubation (as mentioned above) and 100µl of 5 mg/ml MTT dye was added to each well. This plate was incubated for 4 h at 37 °C. The purple-colored Formazan precipitate was dissolved in 150µl of Dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemicals Private Limited, India). The optical density (OD) of each well was measured at 570 nm with a differential filter of 630 nm using a Multiskan Microplate Spectrophotometer (Thermo Scientific™). Cell viability was calculated using the following formula: -

$$\% \text{ Cell Viability} = (\text{OD of treated cells} / \text{OD of Control (without treatment cells)}) * 100$$

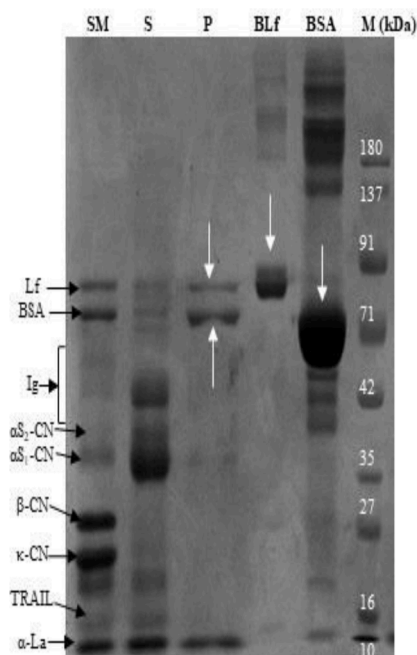
#### 2.10. Statistical analysis

Prism GraphPad version 5.01 was used for the statistical analysis and graphical representation of the data obtained from cytotoxicity assays. Data were expressed as the mean values ± standard error of means (SEM). All experiments were done in triplicates ( $n = 3$ ). One-way and two-way ANOVA followed by Bonferroni post-test at 95% confidence level was used to compare inter and intra groups. Statistically significant differences with respect to control were indicated by \*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\* $p < 0.0001$ , ns = non-significant, respectively. At least 3 experimental repeats were conducted of the other techniques, except LC-MS/MS, which was outsourced, and analysis performed in duplicate.

### 3. Results and discussions

#### 3.1. Analysis of camel milk lactoferrin isolate by SDS-page

The electrophoretic analysis of the sample was performed to identify component proteins present in the lactoferrin isolate and check for its purity. In Fig. 2 lane 1 represents the skimmed milk (SM) prepared earlier. This shows the presence of various proteins like lactoferrin, Camel serum albumin (CSA); Immunoglobulin (Ig);  $\alpha_1$ -Casein ( $\alpha_1$ -CN);  $\alpha_2$  - Casein ( $\alpha_2$ -CN); Beta Casein ( $\beta$ -CN); Kappa Casein ( $\kappa$ -casein); TNF-related apoptosis-inducing ligand (TRAIL and  $\alpha$ -Lactalbumin ( $\alpha$ -LA). Lane 2 shows the protein profile of the supernatant (S) showing the presence of various proteins like CSA; Ig;  $\alpha_1$ -CN;  $\alpha_1$ -CN;  $\beta$ -CN;  $\kappa$ -casein, TRAIL,  $\alpha$ -LA. Lactoferrin is present in the pellet (lane 3). The only other protein present in this lane was serum albumin (which is the most abundant protein in whey). The subsequent lanes are loaded with bovine lactoferrin (lane 4), bovine serum albumin (BSA) in lane 5, and molecular weight marker (Lane 5). The presence of a band in the eluted fractions at the same position as that of bovine lactoferrin was used to identify the protein as lactoferrin. The identity of lactoferrin was further confirmed using LC-MS/MS, as shown subsequently. The molecular weight of the extracted lactoferrin from camel milk samples was estimated to be 78 kDa.



**Fig. 2.** SDS PAGE of the skim milk (SM), Dialyzed Lactoferrin Pellet obtained after 80% Ammonium Sulphate precipitation (P), supernatant obtained after 80% Ammonium Sulphate precipitation (S) along with the marker (M), Bovine Lactoferrin (LF), BSA (Bovine Serum Albumin) for comparison.

SDS-PAGE has also earlier been used to determine the molecular weight and confirm the purity of the isolated lactoferrin. Liang et al. (2011) determined the molecular weight of bovine lactoferrin purified by gel filtration on Sephacryl S-300 and verified its purity by SDS PAGE [1]. Similarly, Le Parc et al. determined the molecular weight of goat milk lactoferrin to be 78 kDa by SDS-PAGE [22]. A study on bioactive proteins in camel milk has been conducted on many proteins including lactoferrin. This study reported variation in the concentrations of this protein in the numerous samples studied [5].

**3.2. LC-MS/MS analysis**

The pellet containing the lactoferrin isolate was subjected to analysis by LC-MS/MS for further characterizing. A high score and coverage indicate a higher probability of its presence. The details of proteins with a score of above 90 have been shown in Table 1. It can be observed that the purified sample included camel milk lactoferrin and camel serum albumin in an intact form (Fig 2.).

LC-MS/MS determination of a sample facilitates the absolute or relative abundance of individual proteins. Protein Score is the sum of the ion scores of all peptides that were identified. Coverage is the percentage of the protein sequence covered by identified peptides. A peptide-spectrum match (PSM) scoring function assigns a numerical value to a peptide-spectrum pair (P,S) expressing the likelihood that the

fragmentation of a peptide with sequence P is recorded in the experimental mass spectrum S. The number of PSM's is the total number of identified peptide spectra matched for the protein. The presence of lactoferrin is confirmed by performing the LC-MS-MS as well as SDS-PAGE (discussed earlier). This suggests that the extraction procedure gives bioactive lactoferrin with very minimal impurity.

**3.3. Cytotoxicity of purified camel milk lactoferrin**

Cytotoxicity of the pellet (P) containing lactoferrin and the supernatant (S) fraction containing other proteins have been shown in Fig. 3 (a) and Fig. 3(b), respectively. It can be seen from Fig. 3(a) that pellet containing lactoferrin was cytotoxic to Hela cells in a concentrations-dependent manner at all the concentrations studied. It was observed that the cytotoxicity increased with the increasing concentration of pellet in a statistically significant manner. Furthermore, the cytotoxicity of lactoferrin against the cells was more at 48 h than 24 h, as expected upon increasing incubation time. Fig. 3(b) shows that the supernatant fraction induced no significant change in cytotoxicity in 24 h (at low concentration), but after 48 h, an improved cytotoxic efficacy was observed. The supernatant may also have other cytotoxic agents (such as TRAIL) besides having trace amounts of lactoferrin. This is portrayed by their cytotoxicity upon prolonged incubation.

The IC50 of the lactoferrin isolate, present in the pellet, was determined to be ~50 µg/ml at 24 h. In contrast, 93.17% has been observed in cells treated with supernatant at the same protein concentration. This confirms the presence of cytolytic lactoferrin in the pellet and not the supernatant. Furthermore, the minor amount albumin that is present in the pellet is known not to be cytotoxic to either normal or transformed cells. Thus, the bioactivity against Hela cells can be totally attributed to the presence of lactoferrin.

Human lactoferrin and bovine lactoferrin have been shown to exert anticancer activity for both tumor prevention and treatment [23, 24]. The tumor preventive effect of lactoferrin has been demonstrated in several animal models bearing different types of tumors such as lung, tongue, esophagus, liver, and colon [25–28]. Lactoferrin treatment was found efficient in inhibiting growth, metastasis, and tumor-associated angiogenesis [29, 30], and in boosting chemotherapy [31, 32]. It has been recognized that human and bovine lactoferrin and their peptide derivative exert a pivotal role in cancer prevention and treatment [36]. Some of the functions exerted by lactoferrin can also be affected by its iron-binding status. It can scavenge free iron in inflamed or infected sites. It does so by suppressing free radical-mediated damage and decreasing the availability of the metal to pathogens and cancer cells. In addition, many studies have shown that, depending on the iron-saturation rate, lactoferrin can exert dissimilar functions by activating specific signaling pathways [33, 34].

None of the current cancer treatment modalities, such as radiotherapy, chemotherapy, immunotherapy, and surgery, are devoid of side effects, and often adversely affect the quality of life of the patients. Recently, the use of functional foods is increasingly being recognized because of their relative safety, immuno-compatibility, bioavailability, low-cost effectiveness, and abundance. The use of chemotherapeutic drugs has given rise to drug-resistant bacterial infections, which can be

**Table 1**  
Mass spectrometric data analysis.

| Accession Number | Description  | Score   | Coverage | #PSMs | #AAs | MW [kDa] | Calc pI | Biological Process  | Cellular Component |
|------------------|--|---------|----------|-------|------|----------|---------|---|--------------------|
| S9WI87           | Serum albumin OS=Camelus ferus<br>OX=419,612 GN=CB1_001109031 PE=4<br>SV=1 | 199.207 | 31       | 170   | 1100 | 125.2    | 5.32    | transport   | extracellular      |
| W6GH05           | Lactoferrin OS=Camelus dromedarius<br>OX=9838 PE=2 SV=1                    | 90.295  | 40       | 47    | 708  | 77.3     | 8.24    | defense response.<br>metabolic process;<br>biological process<br>regulation | extracellular      |



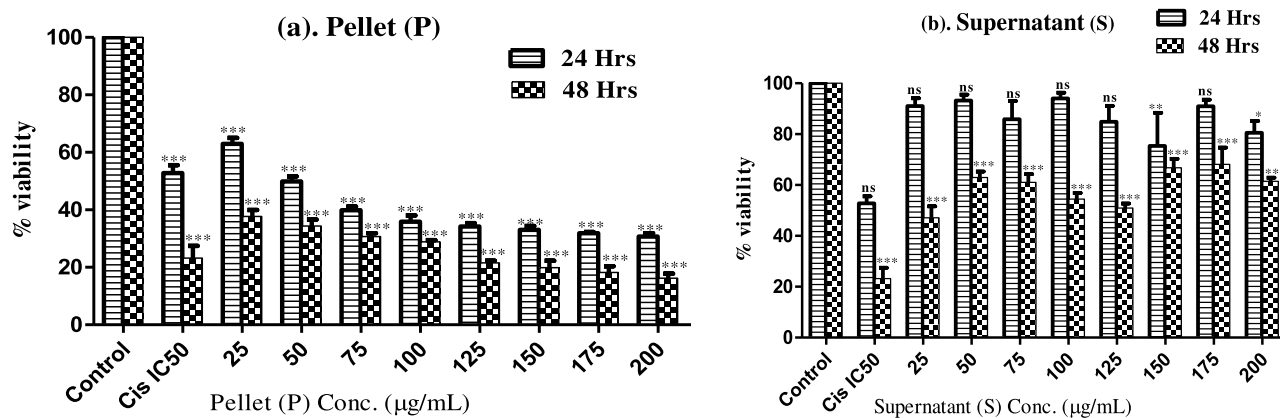


Fig. 3. Cell viability of HeLa cells treated with (a) Pellet containing lactoferrin (P) and, (b) Supernatant (S), obtained from the pH-dependent method at 24 and 48 Hrs. Data are presented as mean values  $\pm$  SEM ( $n = 3$ ).

overcome using lactoferrin powder or tablets as supplementary in addition to chemotherapeutic drugs at optimal concentrations. Lactoferrin, the natural protein, is a highly promising bio-drug in antibacterial therapeutic research. Moreover, the oral route makes it very easy to be administered and is generally well-tolerated. In this context, milk proteins have also been studied earlier as vital nutraceutical ingredients [35]. Furthermore, lactoferrin has to be considered as a powerful weapon against cancer not only due to its ability to prevent and treat cancer but also to boost conventional clinical approaches [36].

#### 4. Conclusion

Camel milk is well recognized for its medicinal properties. Camel milk lactoferrin can serve as a nutraceutical of clinical relevance owing to its antimicrobial, immunological, and anticancer properties. The non-availability of a simple procedure for large-scale purification of camel milk lactoferrin poses a challenge and limits its application. Our present study exemplifies a simple pH-dependent method to purify lactoferrin from camel milk. LC-MS analysis confirmed lactoferrin's presence (sequence length 708aa, molecular weight  $\sim$ 77.3 kDa, and pI  $\sim$  8.24). It is worth noting that the activity was not lost during the pH-dependent method of lactoferrin purification. The importance of this lactoferrin purification method lies in its simplicity and scope of scaling up for large-scale production for commercial or industrial purposes. In future the in vivo studies of the purified lactoferrin can be conducted.

#### CRediT author contribution

**Uma S Dubey:** Conceptualization; Supervision; Writing – review & editing. **Neelam Mahala:** Formal analysis; Investigation; Methodology; Software: Visualization; Writing – original draft. **Aastha Mittal:** Validation; Writing – review & editing. **Manohar Lal:** Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as any conflicting/competing interest.

#### Data Availability

Data will be made available on request.

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#### Further reading

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