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Evaluation of the efficacy of camel milk lactoferrin nanoliposome against colorectal cancer

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ABSTRACT

Colorectal cancer (CRC) is stands as the third most prominent cause of cancer-related mortality globally. Lactoferrin (LF) is a promising molecule that was found to inhibit the growth of cancerous tumors. In the present study, the influence of camel milk lactoferrin nanoliposome (LFnl) on colorectal cancer (HTC-116 cells) as a potential therapeutic agent was investigated. The total purified camel milk LF concentration was 200 mg/l. Film hydration technique was used to produce LFnl. Advanced characterization revealed distinct nanoliposome shapes with a clear two-layered structure. The particle size ranged between 28.3 – 139 nm, and the zeta potential value was -22.6 mV. Exceptional encapsulation efficiency of 99.32% was achieved. The examination of HTC-116 cells and normal cells WI-38 elucidated an IC_{50} value of 40.5±0.9 µg/ml. while, the cytotoxicity assessments conducted on WI-38 cells exhibited a CC_{50} value of 43.5±0.3 µg/ml. This result indicated that LFnl should be used carefully when treated colorectal cancer.

Keywords: Camel Milk, Lactoferrin, Nanoliposome, Colorectal Cancer

INTRODUCTION

Colon cancer, ranking as the second leading cause of cancer-related deaths globally (Ionescu *et al.,* 2023). The incidence rates are notably higher in western nations when juxtaposed with those in Africa and Asia. Recent reports indicate a rising trend in CRC incidence across various Asian and Eastern European countries, a phenomenon believed to correlate with the adoption of western lifestyles and associated risk factors, including unhealthy dietary habits, obesity, and smoking (Roland *et al.,* 2017). Camel milk (CM) has long been attributed with numerous nutritional and medicinal benefits, particularly in relation to its perceived antidiabetic effects, as recognized by various tribes and nations. Despite this traditional acknowledgment, there has been limited understanding of the chemical properties of CM and the mechanisms underlying its potential health benefits. Recent advancements in research have provided a deeper understanding of the chemical composition and distinctive characteristics of camel milk, shedding light on its potential therapeutic properties and offering insights into its mode of action (Mihic *et al.,* 2016 and Singh *et al.,* 2017). Lactoferrin, a glycoprotein belonging to the transferrin family, that binds non-heme iron and is present in the secretions of mammary glands and various exocrine fluids (Cai *et al.,* 2018). Lactoferrin consists of 708 amino acids arranged into two globular lobes in length with a molecular around 80 kDa (Özer and Yaman 2014). The concentration of lactoferrin varies from 0.1–0.3 mg/ml in bovine milk to 2–5 mg/ml in colostrum. In contrast, lactoferrin levels in colostrum and camel milk are found at concentrations of 0.59–5.10 mg/ml and 0.18–2.48

mg/ml, respectively (Abd El-Gawad, 1996; Konuspayeva, 2007 and Recio, 2009). Lactoferrin plays a crucial role in numerous physiological functions, contributing to the regulation of homeostasis and cell proliferation. In addition to its remarkable antimicrobial potency, it exhibits a spectrum of activities, including antibacterial, antifungal, antiviral, and antioxidant properties. Additionally, lactoferrin exhibits immunomodulatory effects and holds potential as an agent with anticancer properties. Its impact on cell proliferation and migration varies, promoting these processes in normal cells and inhibiting them in cancerous cells. Furthermore, lactoferrin has the capability to hinder cancer development by enhancing the adaptive immune response (Al-Numair *et al.,* 2022; Cutone *et al.,* 2020; Siqueiros *et al.,* 2012; Baker and Baker, 2005; Kanyshkova *et al.,* 2001; Lönnerdal and Iyer, 1995). Colorectal cancer is claiming the lives of approximately 10 million individuals annually, constituting over 15% of the total global mortality rate (Pan *et al.,* 2021). Although natural compounds exhibit distinct anticancer and pharmacological properties, their therapeutic application is hindered by low bioavailability and selectivity. To overcome these limitations, various drug delivery systems, including liposomes, nanoemulsions, films, and nanoparticles, are strategically engineered. These systems aim to enhance the targeting precision, pharmacokinetic efficiency, and cellular uptake of anticancer plant constituents, thereby augmenting their overall therapeutic impact (Lagoa *et al.,* 2020). Nanoliposomes, are defined as nanoscale bilayer lipid vesicles, demonstrate a favorable stability profile, ensuring the preservation of their size within the nanometric scale, ranging between 20 and 100 (Khorasani *et al.,* 2018). The objectives of this study were to assess the anticancer activity of camel milk lactoferrin nanoliposome against colorectal cancer (HTC-116 cells) as well as study the cytotoxicity effect LFnl against the normal cell WI-38.

MATERIALS AND METHODS

Camel milk sample collection:

Raw bulk camel milk (*Camelus dromedaries*) was obtained from Sidi Abdel Rahman village, Al- Alamein City, Alex Matrouh Desert Rd.

Chemicals:

CM Sephadex C-50 resins procured from Sigma-Aldrich, located in St. Louis, USA. Soy lecithin insoluble in water was provided from (Noreshark for chemicals Egypt). Mammalian cell lines, including WI-38 cells (human lung fibroblast normal) and HCT-116 (colorectal carcinoma), were purchased from the VACSERA Tissue Culture Unit. Cholesterol was procured from Sigma-Aldrich (St. Louis, USA). Hydrochloric acid (HCl), Sodium hydroxide (NaOH) and Sodium chloride (NaCl) were obtained from El Nasr Pharmaceutical Chemicals Company (ADWIC Egypt). Chloroform was purchased from (Fisher Scientific).

Camel milk chemical composition:

The pH of camel milk sample was determined by using a digital pH meter (Jenway 3510), titratable acidity, total solids, fat content, lactose, and ash were measured according to the accepted methodology in Association of Official Analytical Chemists (AOAC 2019). Protein content was determined through determination of total nitrogen content was measured by the Semi Micro-Kjeldahl method, and the conversation factor was 6.38 (AOAC 2019). Lactose content was determined by using (Foos Milko Scan™ FT2). Each value represents the results of triplicate experiments.

Isolation, identification and determination of lactoferrin:

Camels milk sample was skimmed by centrifugation at 25° C for 15 minutes at 3000 rpm followed by adjustment of skim milk pH to 4.6 using 2N HCL and centrifuged at 5000 rpm for 15 minutes to precipitate casein using (Benchmark centrifuge LC-8 5000).

Lactoferrin was isolated according to (Dionysius *et al.,* 1991) method with some modifications. The whey sample was adjusted to pH 7 using 2N NaOH and then followed by modifying the electrical conductivity value to 15 mS/cm milli-siemens by NaCl powder using (HANNA EC Tester - HI98304) then readjusted to pH 7 by drop wise of 2NaOH.

CM-Sephadex C-50 resins were hydrated by deionized water before use. Five hundred milliliters of whey were added to 50g of hydrated CM-Sepadex C-50 in a beaker and stirred softly using magnetic stirrer (Mtops Ms 300hs) for 60 minutes at 1000 rpm at room temperature.

The mixture was transferred to Buchner funnel which contains hydrated watman filter paper grad 42 installed on filtering flask conducting with vacuum pump and the spent whey recovered by softly vacuum. The resin was subsequently rinsed with approximately 200 ml of deionized water, and then, protein elution was performed using approximately 200 ml 1M NaCl in five steps. To ensure the absence of lactoperoxidase a modification was applied by dialyzing the eluate against deionized water at 4°C for 24 hrs. with several changes of water using dialysis tube (art. D-9777).

Identification of lactoferrin:

Lactoferrin identification was conducted through SDS-PAGE analysis. The purified lactoferrin sample underwent electrophoresis on a polyacrylamide gel containing 0.1% sodium dodecyl sulphate (SDS-PAGE) using the conventional method. The methodology involved protein denaturation through heating for 5-10 minutes in 1% SDS within a boiling water bath before application to the gel. Protein concentration was adjusted to enable the analysis of approximately 10ug of protein (Laemmli, 1970).

Determination of Lactoferrin concentration:

Camel lactoferrin concentration was measured using high-performance liquid chromatography (HPLC). The lactoferrin sample was prepared for HPLC separation with the following conditions: flow rate of 1/min; utilizing Agilent 1100 series equipment (Waldborn, Germany) including quaternary pump (G1311A), degasser (G1322A), thermostated autosampler (G1329A), and variable wavelength detector (G1314A); the column used was Zorbax 300SB C18 column (Agilent Technologies, USA). The injection was conducted at a wavelength of 205 nm to ensure effective separation.

The solvent system comprised 0.1% formic acid with methanol (solvent A) and a mixture of 0.1% formic acid with water (solvent B) in a ratio of 5:95. The injection was performed under ambient temperature conditions.

Preparation of lactoferrin nanoliposomes:

Lactoferrin nanoliposome (LFnl) was formulated using the film hydration method with some modification (Mohan *et al.,* 2016). 1% (w/v) Cholesterol and 1% (w/v) Soy lecithin were dissolved in 100 ml chloroform forming a mixture. The mixture was introduced into a round-bottom flask for the evaporation of the organic solvent under reduced pressure at 60°C using a rotary evaporator. This process led to the formation of a thin lipid layer in the round-bottomed flask. The thin lipid layer was then hydrated with a lactoferrin solution

containing 10 mM phosphate buffer (pH 7.0) under agitation, followed by sonication for 30 minutes. The resulting LFnl was stored under refrigerated conditions until used in experiments.

Nanoliposome characteristics:

Particles size and Morphology:

Particles size and morphology characteristics of LFnl were examined through Transmission electron microscopy (TEM) using (JEM-1400 TEM, JOEL USA)

were analyzed using Transmission Electron Microscopy (TEM) with a JEOL JEM 1400 instrument in the United States.

Surface charge of nanoliposome:

Zeta potential value of LFnl was measured using a Laser Zeta meter, and the results were expressed in millivolts (mV). (Zetasizer Ver. 7.04 Malvern Instruments, UK).

Determination of encapsulation efficiency:

The encapsulation efficiency (EE%) is a metric expressed as a percentage, calculated by determining the ratio of lactoferrin that is encapsulated (L_E) to the total amount of lactoferrin present (L_T). Lactoferrin nanocapsules and nanoliposomes solutions were centrifuged at 30,000 rpm for 15 min followed by withdrawal clear solution to determine (L_E) after excluded precipitated amount which determined by using Lowry methods Lowry *et al.* (1951) as a total protein.

The encapsulated lactoferrin amount was indirectly determined by measuring the nonencapsulated lactoferrin (L_{NE}) using the following equation according to (Jafari *et al.,* 2007):

$$EE\% = L_E/L_T \times 100$$

$$L_E = L_T - L_{NE}$$

Cytotoxicity of camel milk lactoferrin nanoliposome:

The assessment of LFnl cytotoxicity against colorectal cancer (HTC-116 cells) was conducted by seeding cells in a 96-well plate at a density of 1×10^4 cells per well in 100µl of growth medium. After a 24-hour incubation, various concentrations of the experimental sample were applied using a multichannel pipette for serial two-fold dilutions on cell monolayers in 96-well microtiter plates (Falcon, NJ, USA). Incubation at 37ºC with 5% CO_2 followed for 48 hours, with each test concentration represented by three wells. Control cells were subjected to incubation without the test sample, with or without dimethyl sulfoxide (DMSO), the minimal percentage of which (maximum 0.1%) showed no impact on outcomes.

Post-incubation at 37°C, viable cell yield was assessed colorimetrically. After removing the media, a 1% crystal violet solution was added, and after 30 minutes, excess stain was eliminated by rinsing plates with tap water. A 30% glacial acetic acid solution was then added to all wells, mixed thoroughly, and absorbance was measured at 490 nm using a Microplate reader (TECAN, Inc.) after shaking. Results were adjusted for background absorbance in stain-free wells.

Treated samples were compared with the cell control without the tested compounds, with each experiment replicated in triplicate. The cytotoxic effect of each compound was

computed, gauging optical density with a microplate reader (SunRise, TECAN, Inc, USA) to ascertain viable cell count. The percentage of viability was determined using the formula [1 - (OD_t/OD_c)] x 100%, where OD_t represents the mean optical density of wells treated with the tested sample, and OD_c denotes the mean optical density of untreated cells.

RESULTS AND DISCUSSION

Chemical Composition of Camel Milk:

The average amount of components of camel milk is water content 87.1 %; total solids 12.9 %; fat 4.2%; protein 3.5%; lactose 4.45%; ash 0.75%, titratable acidity 0.17 %; and pH value 6.6.

Isolation, identification and determination of camel milk lactoferrin:

About 700 ml of whey protein solution produced after milk fat separation and casein precipitation. 500 ml of this quantity and approximately 10g CM-sephadex C-50 were used to reach to the maximum amount of purified lactoferrin.

SDS-PAGE, or sodium dodecyl sulfate polyacrylamide gel electrophoresis, stands out as a widely employed analytical method for the separation and characterization of proteins. This technique relies on the variance in molecular weight to effectively separate molecules. In the context of this study, the presence of lactoferrin was confirmed through the execution of SDS-PAGE, affirming its utility in protein analysis.

Fig.1. showed presence of a lighter single band around molecular weight 80 kDa, which designated as lactoferrin. This result is agreement with (Redwan and Tabll, 2007) which recorded a single protein band corresponding to lactoferrin (80 kDa).

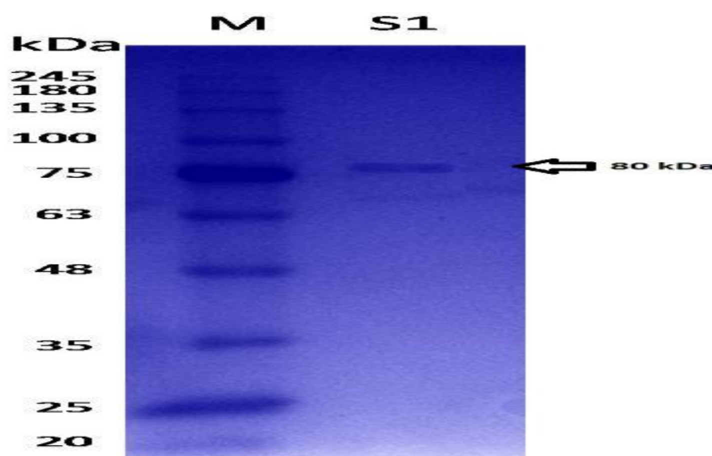

Fig (1): SDS- PAGE of camel milk lactoferrin. A lighter single band around molecular weight of lactoferrin was shown 80 kDa.

The concentration of lactoferrin was determined using High-Performance Liquid Chromatography (HPLC). The standard of lactoferrin used for the optimisation of the HPLC analysis. The concentration of lactoferrin was calculated by peak area integration at 205 nm. The result showed that total lactoferrin was detected at the concentration approximately 200 mg/l Fig.2.

This Average of LF concentration was confirmed with (Król *et al.,* 2012) reported that the average of lactoferrin concentration in milk of different species (mg/l): Human 700-2000, Camel 200-728, Caw 80-500, Buffalo 50-320, Goat 98-150 and Ewe 140.

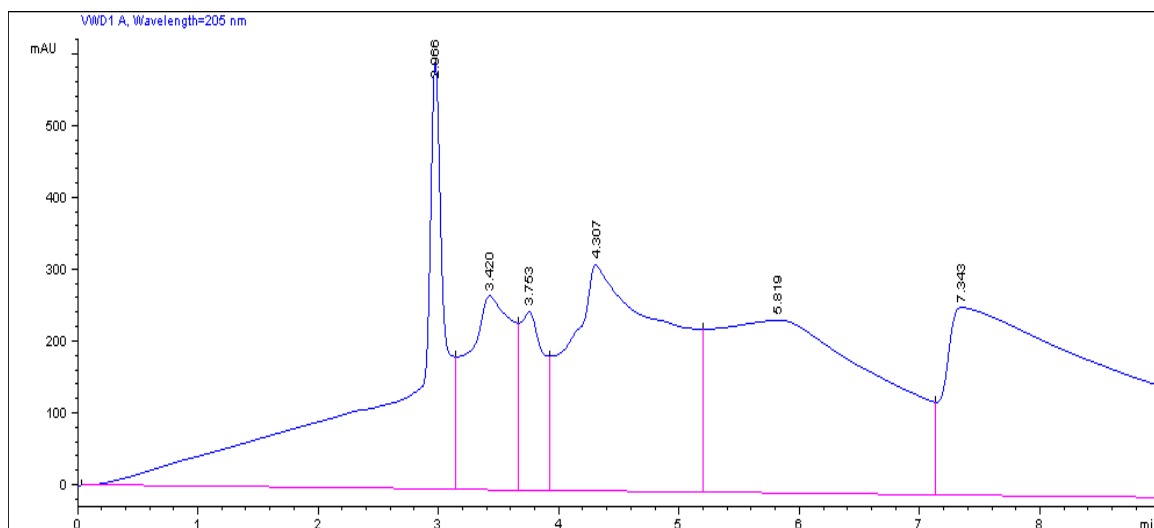

Fig (2): RP-HPLC chromatograms of purified camel milk lactoferrin.

Characterization of camel milk lactoferrin nanoliposome:
Morphology and size of lactoferrin nanoliposomes LFnl:

In Figure 3. TEM images showed that the LFnl have a spherical shape with a practically smooth surface. Lactoferrin nanoliposomes exhibited distinct bilayer structures, whereas certain other particles displayed incomplete structures, marked by the presence of a single layer.

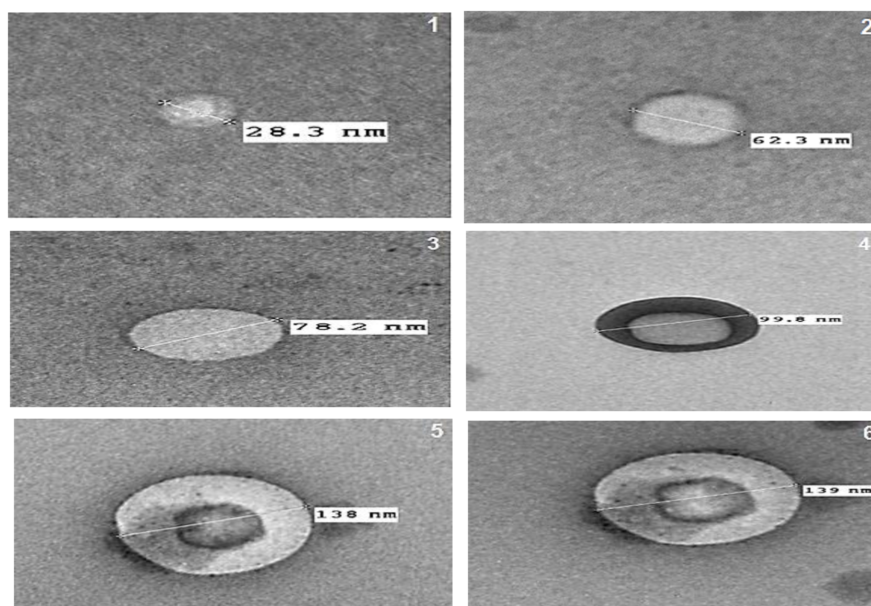

Fig (3): TEM photographs of LFnl using 120000 × and 150000 × magnifications showing nanoliposome shapes and size.

Table (1): Image analysis of TEM photograph shows the particle size of LFnl.

Particle no.	Particle size (nm)
1	28.3
2	62.3
3	78.2
4	99.8
5	138
6	139
Size range	28.3 – 139

Table .1. shows the size analysis of TEM images for LFnl. The particle size ranged between 28.3 and 139 nm, indicating the formation of LFnl in nanoscale particles.

Zeta potential:

Zeta potential (ζ-potential) is a physical property which used to measure of the electrical charge of particles that are suspended in liquid. Colloidal system least stable at zero zeta potential which called isoelectric point. Zeta potential value will be positive at low pH and negative at high pH.

Zeta potential value of LFnl was determined at pH 7, resulting in a negative value of -22.6 mV. This value is better than the value reported by (Raei *et al.,* 2015), who indicated a zeta potential range between (-5.036) to (-5.111) mV for non-thermal lactoferrin nanocapsules from camel milk. Huang *et al.* (2013) reported that zeta potential value for lactoferrin-conjugated liposomes as a novel carrier was −0.60 ± 0.51 mV which agree with our findings of LFnl zeta value.

Clogston and Patri (2011) reported that nanoparticles with a zeta potential within the range of -10 and +10 mV are considered approximately neutral. In contrast, nanoparticles with zeta potentials exceeding (+30) mV or less than (-30) mV are classified as strongly cationic and strongly anionic, respectively.

Based on our findings, the lactoferrin nanoliposome is considered to be characterized by a high degree of stability due to the high negative value, which means the success of the film technology in producing lactoferrin nanoliposomes with the required specifications. The observed result can be attributed to a comparatively elevated surface charge, leading to pronounced electrokinetic repulsion and, consequently, heightened stability in the suspension.

Encapsulation efficiency:

The encapsulation efficiency (EE%) is used as a crucial metric for assessing the efficacy and viability of the preparation methods. EE% was calculated by equation after determined the concentration of nonencapsulated lactoferrin. The EE% of LFnl was 99.32 % that means 99.32% of the initially purified lactoferrin during the encapsulation process was successfully incorporated into the nanoliposomes. Our result is highly confirmed with (Raei *et al.,* 2015) who reported that the EE% was 100 % for lactoferrin when using the ionic gelation method. Guan *et al.,* (2012) prepared LFnl using also film method. The EE% was 48.9 % which means approximately 51.1% of lactoferrin only incapsulated compared with our results, it may lead to the using of sonication during preparation nanoliposome enhancing the encapsulation efficiency than using the homogenizer.

Cytotoxicity of Nanoparticles:

Cytotoxicity refers to the detrimental effects arising from interactions with essential cellular structures and processes vital for cell maintenance, encompassing aspects such as proliferation, survival, and normal biochemical and physiological functions (Sittampalam *et al.,* 2004).

Cell culture models are suitable for assessing the toxicity of various substances, including nanomaterials. Information derived from these models can offer insights into the safe use of such particles in humans, especially considering their established hazardous nature (Melo *et al.,* 2014).

Utilizing cell culture models provides a significant advantage in minimizing the potential alterations that could occur in vivo experiments, particularly under experimental stress. Numerous cell culture models and in vitro cytotoxicity assays are accessible for conducting experiments in this domain. It is crucial to comprehend both the advantages and limitations of each model to ensure the acquisition of dependable results (Freshney, 2000).

In conclusion, cultured cells play a versatile role in exploring diverse facets of biochemistry and functional cell biology. They are utilized to assess the effects of pharmaceuticals on cell physiology, unravel the pathways and processes involved in cell death and aging, conduct nutritional studies, and investigate the interactions between disease-causing substances and cells (Freshney, 2000; Machana *et al.,* 2011; Pintus *et al*., 2012; Fukazawa *et al.,* 2012 and Acosta *et al.,* 2013).

Viability tests are utilized to measure the percentage of viable cells in response to immediate or short-term changes, including alterations in membrane permeability or shifts in specific metabolic pathways associated with cell survival and proliferation. This viability assay specifically targets living cells capable of incorporating the dye and sequestering it within lysosomes. Analyzing variations in neutral red uptake is essential, as these changes may indicate potential toxicities associated with lysosomal disorders in organs like the liver, kidney, and lung (De Duve *et al.,* 1974; Schneider *et al.,* 1997 and Stern *et al.,* 2012).

The cytotoxicity of LFnl against HCT-116 and WI-38 cell line were applied to evaluate inhibitory effect. The 50% inhibitory concentration (IC_{50}) or cell cytotoxic conc. (CC_{50}), representing the concentration required to induce toxic effects in 50% of intact cells, was determined by analyzing dose-response curves for each concentration using software system (GraphPad Software, San Diego, CA, USA).

Human colorectal carcinoma cell line (HTC-116):

The data presented in Table 2 and Fig. 4 confirm the inhibitory effect of LFnl against colon cancer cells. The IC_{50} value of LFnl against HTC-116 cells was determined to be 40.5±0.9 µg/ml. This result aligns with the findings of (Habib *et al.,* 2013), who reported that LF inhibited the growth of colon cancer cells (HTC-116).

This mentioned result agrees with our findings; however, it's noteworthy that the concentration of LFnl we used was lower than their dosage. This suggests a comparative advantage in utilizing lower LFnl concentrations for the treatment of colorectal carcinoma.

Ma *et al.,* (2013) investigated the effect of LFnl against other model of a colorectal carcinoma cell line. They reported that the viabilities of Caco-2 cells decreased with LFnl at concentrations of 5 and 10 mg/ml.

The efficacy of LFnl against colorectal cancer cells is substantiated by a study conducted by (Yao *et al.,* 2021). The research demonstrated that the combination of lactoferrin and linolenic acid effectively inhibited the colorectal cancer cell line (HT29 cells) by activating the AMPK/JNK-related apoptosis pathway.

Anticancer activity of lactoferrin nanoparticles against human colorectal carcinoma (Caco-2 cell lines) was confirmed by (El-Fakharany *et al.,* 2023) who demonstrated that the innovative nanocombination of apo-lactoferrin selenium nanocomposites (ALF-Se NP_s) exhibits enhanced selectivity and apoptosis-inducing anticancer activity when compared to free apo-lactoferrin or individual forms of selenium nanoparticles.

Table (2): The inhibitory effect of LFnl against HTC-116 cell line.

Sample conc. (µg/ml)	Inhibitory %	Viability %
0	0	100
1.56	0.52	99.48
3.125	4.79	95.21
6.25	10.27	89.73
12.5	19.04	80.96
25	32.76	67.24
50	60.47	39.53
100	71.24	28.76
200	79.07	20.93

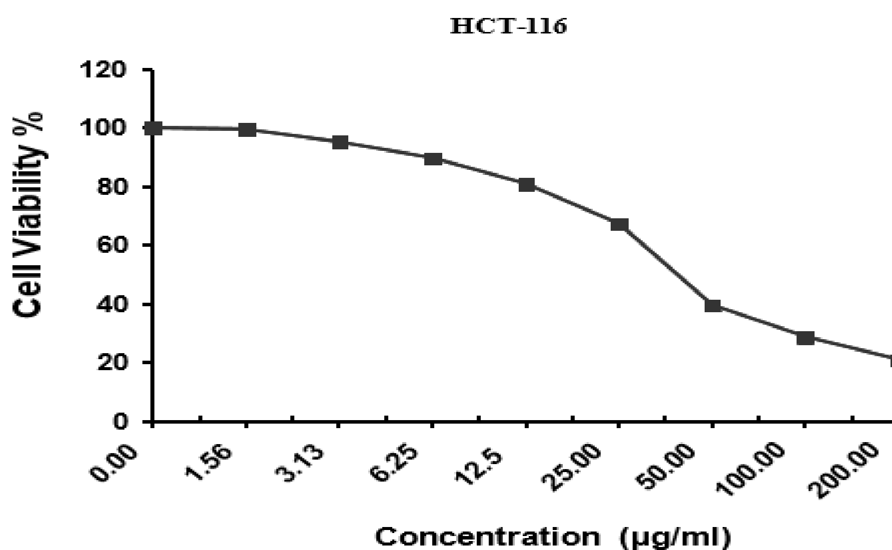

Fig.4. The relation between LFnl concentration and the level of HTC-116 cell viability.

Human Lung Fibroblast normal cell (WI-38):

The cytotoxicity of LFnl was also investigated to assess the inhibitory effect of different concentrations against human lung fibroblast normal cells (WI-38 cell line). As shown in Table.3 and Fig. 5. the CC_{50} was achieved at 43.0 ± 0.3 µg/ml. When compared with

the IC_{50} values for HTC-116 (40.5 ± 0.9 µg/ml), this result indicates that LFnl should be used cautiously in the treatment of colorectal cancer.

Table (3): The inhibitory effect of LFnl against to WI-38 cell line.

Sample conc. (µg/ml)	Inhibitory %	Viability %
0	-	100
1.56	0	100
3.125	2.96	97.04
6.25	10.59	89.41
12.5	21.77	78.23
25	39.28	60.72
50	58.11	41.89
100	70.55	29.45
200	81.24	18.76

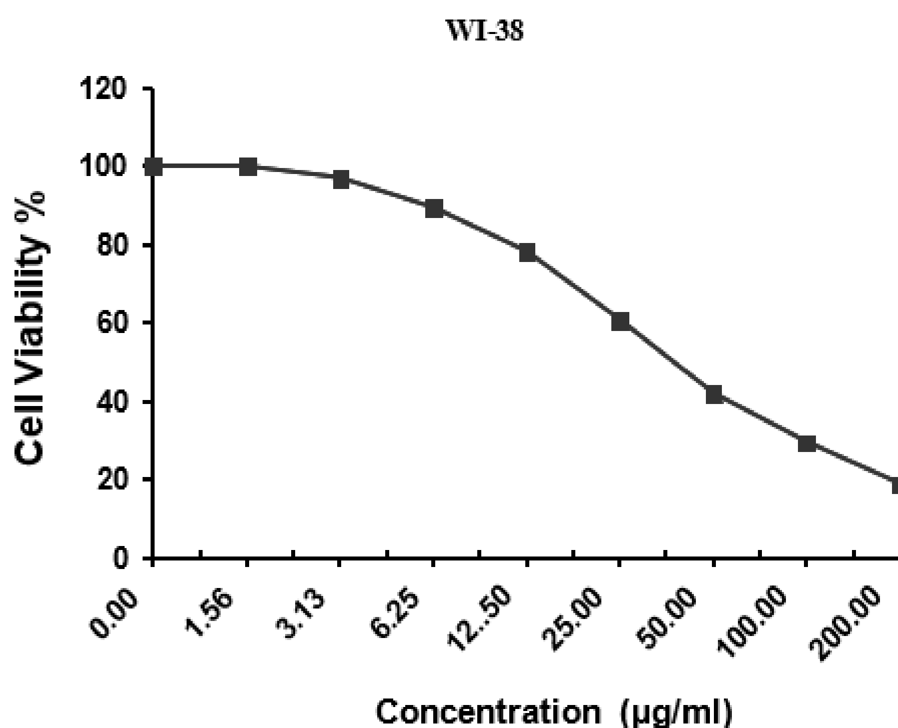

Fig.5. The relation between LFnl concentration and the level of WI-38 cell viability.

Conclusions:

The efficacy of camel milk lactoferrin nanoliposomes against colorectal cancer cells demonstrates a significant inhibitory effect on the tested cells, presenting a potentially promising approach for impeding the growth of malignant tumors. Nonetheless, further comprehensive studies, particularly *in vivo* investigations, are imperative to validate this effect and ascertain the safety profile of this compound prior to its clinical application in cancer treatment.

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