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Research Paper

Loading lactoferrin drived from Camel milk on the pectin nanoparticles and studying the its effect on the MCF-7 cancer cell line

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A B S T R A C T
Due to high prevalence of cancer in the current society, finding novel drugs for its treatment is vital. The main purpose of this paper was to study the effect of anti-cancer of lactoferrin loading on the pectin nanoparticles. In order to enhance the effectiveness of lactoferrin on the cancer cells, the loading on the pectin nanoparticles has been utilized. Formation of lactoferrin complexes at final volume of 0.2% with pectin nanoparticles at three concentrations of 0.01, 0.1 and 0.5% and three PH levels of 4, 5.5 and 7 with 1:1 ratio was carried out.
The results of this experiment showed that the lowest zeta potential and the smallest size particles in the concentration of 0.5% pectin with lactoferrin exist in PH=7. In addition, the highest and lowest loading rates in nanoparticle complexes were obtained at concentration 0.01 % of pectin with lactoferrin in PH=4 and concertation 0.1% pectin with lactoferrin in PH=5.5. the highest emission reduction was due to the concentration 0.5 % pectin with lactoferrin. The effect of lactoferrin loaded on the pectin nanoparticle upon the MCF-7 cancer cell lines using MTT experiment was studied and the results showed that binding the lactoferrin to pectin cause to enhance the anti-cancer effects of it. The highest effect was found at the 0.5% concentration of pectin with 0.2% lactoferrin.

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I. INTRODUCTION

Lactoferrin (LF), also known as lactotransferrin (LTF), is a multifunctional protein of the transferrin family (1,2). Lactoferrin is a globular glycoprotein with a molecular mass of about 80 kDa that is widely represented in various secretory fluids, such as milk, saliva, tears, and nasal secretions (3). One of the most important sources of LF is milk (LF is a whey protein) which is simply accessible in some of species milk. For example, the concentration of LF in camel, human, and bovine milks is 2.00– 6.00, 1.00–5.00, and 0.10–0.40 mg/mL, respectively (3). Without considering the main biological function of lactoferrin which is binding and transport of iron ions, it also has antibacterial, antiviral, antiparasitic, catalytic, anti-cancer, and anti-allergic functions. But, because of sensivity in LF against environmental or process stresses, its activities will get restricted. Therefore, the stabilization of LF is necessary for optimal utilization and delivery of LF into body organs (4).
Recently encapsulation of bioactive compounds in nutrition industry, pharmacy process and biotechnology have been beneficial in high range (Jafari et al 2008). Spray drying and electro hydrodynamic processes are the most hopeful and encouraging encapsulation technologies for entrapping and effectively delivering bioactive compounds (5,6). More recently, nano-encapsulation systems (less than 1.0 mm) have been considered for the

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protection and delivery of bioactive compounds due to a lot of advantages like enhanced bioavailability, greater solubility and better permeability (7, 8). Nano-particles (based on complex formation between biopolymers) is produced by the electrostatic interaction of proteins and polysaccharides (9,10). Formation of biopolymer compounds can potentially lead to variable functional traits for example solubility, surface activity, compatibility stability, gelation ability, emulsion and foam properties (11, 12). Consequently, encapsulation of bioactive compounds through nano-particles can provide improved functional properties.

The vital step in producing nano-particles is using the suitable wall materials. Two groups including 1. Proteins (such as as β-lactoglobulin, zein, gelatin, soy protein, collagen, and albumin) and 2. polysaccharides (including pectin, alginate and chitosan) are the momentous biopolymers which can be used for production of complexed nano-particle carriers (11,12). The association of Whey protein which is a natural emulsifier and stabilizer with polysaccharides makes a complex with thickening and steric stabilizing behaviors (13). For example, pectin is one of the most prevalent polysaccharides which has been extensively practical in a complexed form with whey proteins for producing multilayer encapsulation systems (14,15). Scientists have used pectin as a gelling, thickening and stabilizing organ which is widely abundant in plant cell walls especially from apple pomace, citrus fruits, and sugar beet root. Having considered pectin a family of galacturonic acid-rich polysaccharides including, rhamnogalacturonan I, homogalacturonan, and the substituted galacturonan rhamnogalacturonan II, and xylogalacturonan (16). The conclusion is that nano-particle complex of whey protein/pectin can be a good candidate for encapsulation of bioactive compounds.

Up to now, a successful remedy for complete control of cancerous masses has not been known. Domines et al (1998) examined the usage of lactoferrin in high concentrations in cancer progression and reported that lactoferrin has an important effect on the cytotoxic activity of natural lethal cells in the lines of the epithelial cells of the breast and hematopoietic(17). Some researchers also demonstrated the effectiveness of lactoferrin on the expression of 10 genes associated with apoptosis in cancers colon cells in rats (18). According to the anticancer effect of lactoffein and its sustainability and efficiency in the present of pectin In the present study, the possibility of using lactoferrin and pectin simultaneously in the formation of electrostatics complexes and its effect on cancer cells (MCF7) has been investigated.

II. MATERIALS AND METHODS

From dromedary camels (Camelus dromedarius), raw milk was prepared in Turkman Sahara (Goleston province, North of Iran) and in refrigerator, it was saved until test time. The Lactoferrin in camel milk was extracted and purified based on previous researches (19).

Then High-Methoxyl Pectin (HMP) was bought from merck (Darmstadt, Germany) (36% esterified, the molar mass of 100 kDa) solvents, reagents and some other used chemicals got graded analytically and were bought from Merck (Darmstadt, Germany).

Biopolymer solution preparation

Purified lactoferrin and pectin powders were weighed into separate beakers for make soluble. (20). So solutions were prepared by adding double-distilled water and stirring constantly at ambient for 2 hours and kept overnight in the refrigerator. Therefore, Lactoferrin solution with concentration 0.2 % (w/v) and three pectin concentration (0.01%-0.1%-0.5 %) (w/v) in pH values (4.0, 5.5 and 7) were prepared and used for complex lactoferrin/pectin.

Preparation of complex lactoferrin/pectin nano-particles

For complexes preparation, at first we need to provide a solution at a concentration 0.5, 0.1 and 0.01 % of pectin (w/v), then with gentle magnetic stirring for 2 hours at 400 rpm at ambient and kept overnight in the refrigerator to ensure full encapsulating. Lactoferrin entrapment was done by combining (0.2% w/w) lactoferrin into HMP solution followed. with gentle magnetic stirring for 2 hours at 400 rpm at ambient and kept overnight in the refrigerator to ensure full encapsulating.

2.3. Zeta potential and particle size measurement

The zeta potential and particle size of lactoferrin and HMP complexes were measured by devices (CAS Instruments, France, VASCO-1, France). For more precision, the solutions were first diluted.

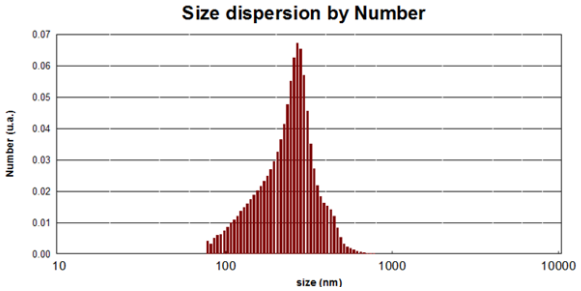


Fig. 1. Droplet size analysis results (by a dynamic light scattering (DLS) technique) of complex nano-particles Lf-HMP 0.5%, pH= 5.5

Entrapment and encapsulation efficiency:
Complex-entrapped LF was defined by HPLC. Lactoferrin was transfused into column C18, HPLC (Agilent, series 1200). By a UV-visible detector of 280 and 320 nm, Lactoferrin was discovered and compared to a calibration curve for pure LF.So efficiency for entrapment (encapsulation) was measured as below:

$$\text{EE } \% = \frac{(C_t - C_f)}{C_t} \times 100$$

Where C_f is the protein amount of unbanded peptide and C_t is the total protein amount of peptide added to nanoparticle.

Morphology study:
All of the chosen specimens were used for microstructural analysis using scanning electron microscopy (SEM). In condition of SEM study, some nano-particles were diffused onto a two-sided adhesive tape and after that covered with a tender layer of gold.The morphological property of particles were revealed by a field emission scanning electron microscope (VP 1450., LEO, Germany).
Tryptophan Fluorescence Spectroscopy study:
The microstructure analysis of the samples was examined by Tryptophan Fluorescence Spectroscopy (Model 2500, Hitachi, Japan). By providing Fluorescence study, it can be considered as a sensitive and easy to use this method.
The effect of lactoferrin and pectin complex on the MCF-7 breast cancer cell lines
In order to study the effect of lactoferrin and pectin complexes on growth of the MCF-7 breast cancer cell lines, 96-well plates have been utilized. MTT experiment had been carried for quantitative study of the cells.
Statistical analysis
In order to compare the measured parameters including zeta potential and mean viability percent of the studied cells (MCF-7 cell line) and to determine the statistical significant differences, Minitab software was used. The significance level has been taken into account at P≤ 0.05. The statistical analysis of experimental data and ANOVA data were carried out by the software.
Cell culture and complexes lactoferrin and pectin incubation
From the American type culture collection, MCF-7 were acquired. For routine culture, media were supplemented with 10% fetal bovine serum (Gibco 10270), and the cells were conserved at 37°C in atmosphere providing 95% air and 5% CO2. For this cure, the cells were permitted to stick after sending (21).
Because of study the effect of lactoferrin-pectin in growing the cancer cells, three concentrations of pectin with three PH levels were used. MCF-7 cell line were conserved at 96-well plate along with 200µL culture medium including 10% and 95% atm air with 5% CO2 one day before adding treatments (they were kept at incubator for 24h because of making certain for cell binding). As soon as finishing the process of treatments, the plate containing cells was conserved at Co2 incubator and after 24h, the amount of viability in the cells were under the control in the culture medium through MTT experiment (21).
Cell viability assay
At a concentration of 2.5 mg/mL in phosphate-buffered saline, the solution was prepared and 500 µL was added per well. Before adding lysis buffer, the solution were removed. Then the optical concentration was measured 1h later at 570 nm due to using a microplate spectrophotometer (Bio-Rad x Mark Microplate spectrophotometer) and microplate manager 6 software (21).

III. RESULTS AND DISCUSSION

Zeta potential and size of LF-HMP nano-particles complexes
The zeta-potential and mean of nano-particles complex is shown in Fig 2. Our results revealed that in different concentrations of pectin (0.01%-0.1%-0.5 %)(w/v), PH shifting from 4 to 5.5 and then 7 was led to a significant

reduction in the Zeta potentiol. ($p \leq 0.05$) Hence, it can be concluded that PH=7 at three experimented concentrations of pectin has the most negative zeta potential. Moreover, it was showed that the most negative zeta potential in the current experiment was related to pectin 0.5% and PH=7.

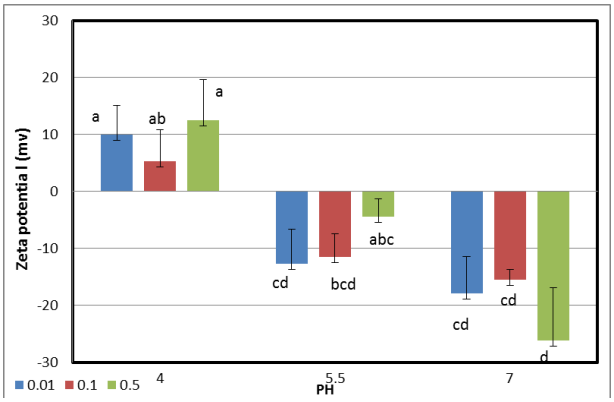


Fig. 2. Zeta potential (mV) of LF–HMP nano-particles ($p \leq 0.05$)

At pH 7 the biopolymer solutions were mingled, due to its value is vaguely lower than the reported isoelectric point of lactoferrin (pH =8) and protein molecules should be charged. Typically, the behavior of individual polymers is affected by the process of other biopolymers in solution when are mingled (22) .

Table 1. Size (nm) of LF–HMP nano-particles

The average particle size(nm)	The average and (percentage of) particle size 1-100(nm)	The average and (percentage of) particle size 900-4000(nm)	The average and (percentage of) particle size 400-900(nm)	The average and (percentage of) particle size 1-400(nm)	The sample (concentration%, PH)
587.8	93.41 (3%)	955.5(3%)	558 (33%)	249.9 (64%)	lf+pe (0.01%, ph =4)
694.38		1237.4 (8%)	569.4 (41%)	276.2 (51%)	lf+pe (0.01%, ph =5.5)
590.4	89.2 (3%)	964.8 (3%)	558 (33%)	248.4 (64%)	lf+pe (0.01%, ph =7)
752.3		1337.6 (27%)	602.6 (51%)	316.7 (22%)	lf+pe (0.1%, ph =4)
799.6		1456.4 (30%)	614.4 (48%)	328 (17.7%)	lf+pe (0.1%, ph =5.5)
708		1230.2 (17.5%)	587.6 (47%)	306.2 (35.5%)	lf+pe (0.1%, ph =7)
656.6		1107.5 (10.8%)	575.2 (42%)	287.3 (47.2%)	lf+pe (0.5%, ph =4)
567.5	81.8 (9%)	1035.7 (2%)	448.4 (8%)	218.5 (90%)	lf+pe (0.5%, ph =5.5)
280.8	67.1 (51%)	1036.8 (1%)	427.7 (2%)	133.9 (97%)	lf+pe (0.5%, ph =7)
596.8		936.7 (2%)	545.4 (53%)	308.2 (45%)	LF (0.2%)
294.8	61.3 (65%)	1069.1 (1%)	487.7 (3%)	101.9 (96%)	PE (0.1%)

It is proved that in a lower pH value, there will be an increasing degree in the positive charge of lactoferrin and consequently, the negatively charged molecules of pectin interact with highly positive charge surface of lactoferrin which since makes complex particles with a small size (23).

The nanoparticles with small size have been produced at 0.5% of pectin with lactoferrin at PH=7. In the Bangochi et al 2011 researches, the potential of zeta for concentration of pectin 0 – 0.15% is variable in the range of (-47.7 to +3.16). in the test, the amount of zeta potential has been more negative because of pectin addition. In the Raei et al 2017 researches WPI/HMP complexes as a transfer for lactoferrin with proportion 1:1 in different PH (3, 3.5 and 4), zeta potential was reported to be equal to -17, -35 and -23 respectively. In the same research the size of particles with ratio 1:1 in PH 3, 3.5 and 4 has been in order 600, 670 and 750 nm. Other studies also have shown that by adding pectin into lactoferrin, the zeta potential has been more negative and furthermore, by comparing PH =7 with other PH the amount of zeta potential got more negative and the size of particles were smaller.

Encapsulation efficiency

By preparing different treatments, encapsulation efficiency in LF/HMP complex nano-particles is shown in Fig 3. The encapsulation efficiency in the highest and lowest ranges in complex nanoparticles was observed in LF-HMP with concentration 0.01 % pectin at pH=4, and concentration 0.1% pectin pH=5.5, respctievely.

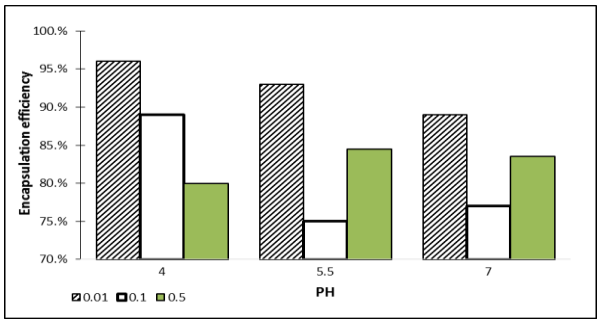


Fig 3. encapsulation efficiency of LF–HMP nano-particles

In order to ensure the purity of lactoferrin, lactoferrin extracted in the experiment was compared to the standard lactoferrin and as the fig 4 shows, the lactoferrin extracted in our experiment shows a similar standard lactoferrin of a peak which indicate the purity of the extracted lactoferrin.

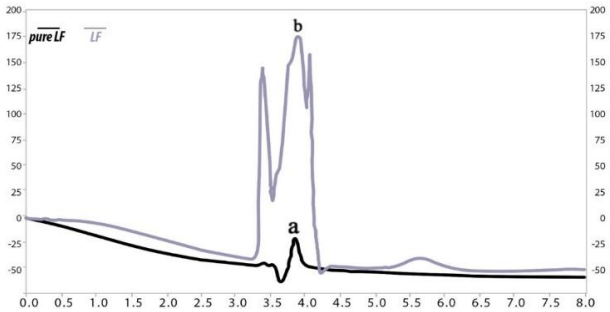
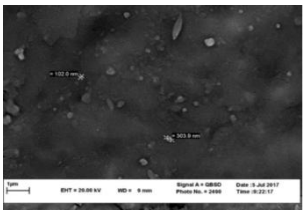


Fig 4. The HPLC graphs of the (a) pure LF and (b) LF peak of lactoferrin extracted by ion-exchange chromatography method

3.3. Morphology and analysis of nanocomplexes loaded with LF

By means of SEM, the morphology of LF-HMP nanocomplexes (with a ratio of 1:1) was examined. It was uncovered that the nanoparticles had globular shapes with nano-scale size and presented a wide range of particle sizes. In addition, there was an outstanding similarity in distribution of nanoparticles obser size in nanoparticles which was noticed in SEM images.



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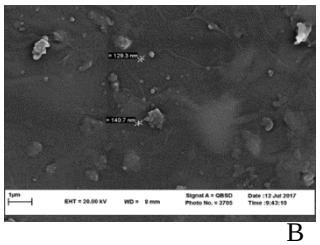

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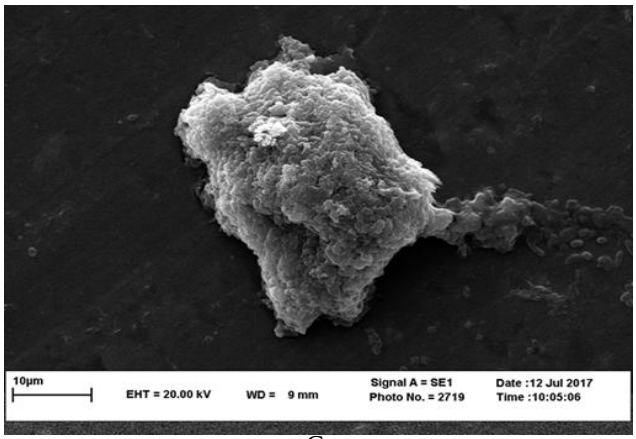

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Fig 5. (a) SEM of LF and (b) SEM of pectin, and (c)SEM of nano-particles produced by LF-HMP complex at a ratio of 1:1.

Tryptophan Fluorescence Spectroscopy

Each chart shows the spectrum of the intensity of fluorescence emission related to lactoferrin protein individually and the spectrum of the intensity of fluorescence emission related to nanoparticle banded protein. In this spectrum, it is evidently observed that lactoferrin protein has a strong fluorescence emission at 322nm wavelength. Through adding lactoferrin protein to prepared nanoparticle, the significant decrease at the intensity of fluorescence emission was found that this process is called as fluorescence quenching.

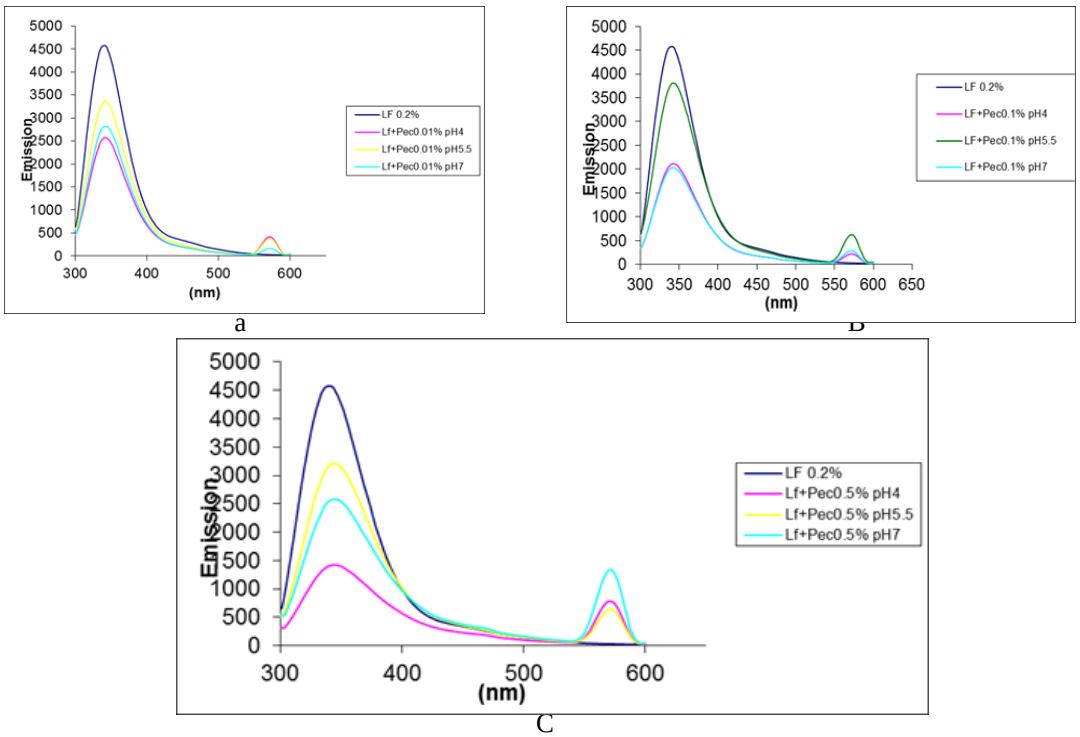

Fig 6. Tryptophan Fluorescence Spectroscopy.
(a)density 0.01% pectin (b) density 0.1% pectin (c) density 0.5% pectin

Lactoferrin protein with 0.2% concentration has a strong fluorescence emission at wave length of 332nm. Through adding Lactoferrin protein to pectin nanoparticle with 0.01 concentration, the percentage of reduction in the intensity of fluorescence emission or the same process of fluorescence quenching was found. Since the effect of PH is considered as a factor affecting on fluorescence emission, at 0.01% concentration of pectin with 0.2% lactoferrin, The PH=4 shows the highest reduction in the intensity of fluorescence emission.

At concentration of 0.1% pectin with 0.2% lactoferrin, PH=7 shows the highest reduction in the intensity of fluorescence emission. At concentration of 0.5% pectin with 0.2% lactoferrin , PH=4 shows the highest reduction in the intensity of fluorescence emission.

If the rate of reduction in the intensity of fluorescence emission at three concentrations of pectin (0.01%,0.1%,0.5%) with 0.2% lactoferrin will be studied, this point should be considered that one of factors affecting on the intensity of fluorescence emission is concentration effect. Based on the Beer-Lambert law, arguably the intensity of fluorescence depends to the number of particles which show the fluorescent behavior in the dissolved form. Therefore, the intensity of fluorescence is directly proportional to material concentration.

Through comparison of three charts, it is found that highest reduction in the intensity of fluorescence emission is related to 0.5% pectin. At 0.5% concentration of pectin at three PH levels, the intensity of fluorescence emission is reduced more than other two concentrations.

Cell viability assay

The studies have shown that growth restrain of cancerous cells by lactoferrin may have been in connection with the ability of that for apoptosis injection by activating the signaling proses Fas (18). In fact, LF by blocking the epithelial function and reducing of interlukin 18 production act as a anjiojenic regulator.

This information suggest that was plays and inhibitive role in the progression of breast cancer cells. Thefore in can be used as a therapeutic protein in tumorigenic breast cancer (24). They also proved that wap reduces the progression of human breast cancer cells.

The percentage of cell viability with increasing the pectin concentration and the effect of PH was shown

in fig 7 . Lactoferrin contributes less to cell death but when lactoferrin protein has been loaded on the pectin particles, the positive and synergic effect is shown on the death of MCF-7 cells line. Due to this chart, the percentage of cell viability and the results of the fluorescence spectrometry and zeta potential, it could be concluded that the optimum is 0.5% pectin with 0.2% lactoferrin at PH=5.5.

We conclude from the statistical out comes that different PH affect the result also different concentration have effect on tested samples but PH and concentration don't interact with the sample.

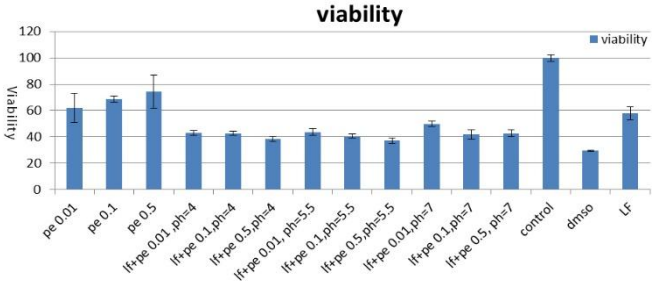

Fig 7. The viability of cancer cells when the pectin concentration increases.

IV. CONCLUSION

The results of this study showed that LF can be loaded in the high methoxyl pectin nanoparticles at pH values of 4, 5.5 and 7.

The smallest particle size was provided by applying LF-HMP complex with concentration 0.5% pectin at pH=7 at ratio of 1:1.

Binding of lactoferrin to pectin nanoparticles was confirmed by tryptophan fluorescence. Average diameter of nanoparticles and Zeta potential were measured before and after attachment of pectin and lactoferrin and at three concentrations and three PHs which these result support and confirm the attachment of pectin and lactoferrin. By Scan electron/production phase and attachment of pectin and lactoferrin is very homogenous with the uniform distribution of diameter. The effect of nanoparticles loaded on the MCF7 cancer cell lines was studied using MTT experiment. The results showed that pectin-lactoferrin has a positive effect on the death of cancer cells and by increasing the concentration of pectin, this effect is strengthening.

In general, our results introduced the nano-particles prepared by LF-HMP complex at a ratio of 1:1 in pH= 7 as an optimal nanocarrier for LF which can be used in the food formulations and pharmaceutical industries.

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