DOI : 10.35124/bca.2019.19.S1.2661

Biochem. Cell. Arch. Vol. 19, Supplement 1, pp. 2661-2667, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

EFFECT OF PURIFIED GOAT MILK LACTOFERRIN ON CANCER CELL GROWTH (AMN-3) *IN VITRO*

Zainab H. Abbas[1], Kifah S. Doosh[2] and Nahi Y. Yaseen[3]

[1]College of Agriculture, Karbala University, Krebala, Iraq.
[2]College of Agriculture, Baghdad University, Baghdad, Iraq.
[3]Iraqi Center for Caner and Medical Genetic Research, Al-Mustansiriyah University, Baghdad, Iraq.

(Received 29 March 2019, Revised 22 May 2019, Accepted 30 May 2019)

ABSTRACT : Lactoferrin is an important protein in many biological applications asa potential cancer treatment agent. Lactoferrin (LF) is a glycoprotein with a molecular weight of about 80 kDa. Due to the size and construction, it belongs to the transferrin family, which has a specific ability to bind iron (Legrand *et al*, 2008). It occurs as a single polypeptide chain which consists of about 690 amino acids (Baker and Barker, 2005). In this study, lactoferrin was purified from goat colostrum by Ion exchange chromatography by using CM-Sephadex G-150 column and gelfiltration by using Sephadex G-200 column. To know its ability as anticancer agent the study utilized an *in vitro* evaluation for the cytotoxic effect of the purified goat milk lactoferrin (gLf) on two cell lines, AMN-3 (Ahmed-Mohammed-Nahi, 2003) cell line and Rat Embryo Fibroblast (REF) cell line at different concentrations and different exposure time of treatment. The purified gLf concentrations ranging (19.53 to 5000) µg/ml for 24, 48 and 72 hours. The effect of gLf was evaluated by employing MTT assay. The results revealed significant cytotoxic effect at levels (P<0.05) for all concentrations and for all exposure time of gLF on RD cell line as compared to untreated control cells. The inhibition rate IR% increased with raising of gLF concentration and incubation period. The highest inhibitory growth was at the concentration (5000 µg/ml) after 72 hrs of exposure time (56.14%), while only the highest concentration gave significant inhibitory effect (P< 0.05) with normal cell REF. The possibility of getting good amounts of lactoferrin from goat colostrum through the use of ion exchange chromatography by using CM-Sephadex-C 50 then gel filtration by Sephadex G-200.

Key words : Goat lactoferrin, AMN-3, REF, MTT assay.

INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells cancer, in which normal cells start multiplying uncontrollably ignoring signals to stop and accumulating to form a mass that is generally termed a tumor (AACR, 2013).

Cancer is the second leading cause of mortality worldwide as it still takes millions of people lives every year around the world. In 2008, almost 12.7 million people were diagnosed with cancer and more than 7.5 million of them were dead (Siegel, 2012). The world health Organization (WHO) estimated that if unchecked, annual global cancer deaths could rise to 15 million by 2020 (Rastogi *et al*, 2004). Recently in Iraq there is a terrible number of unpublished cancer cases beside the published cases by the Iraqi Cancer Council in 2008 were 19.9 thousand and more than 15.4 thousand of them were died (IARC, 2011).

The current conventional cancer treatment options for localized tumors and advanced disease are typically associated with risks and side effects (Ronald *et al*, 2010). The discovery of anticancer drugs remains a highly challenging endeavor and cancer a hard-to-cure disease (Hatzimichael, 2013). The new protocols for cancer therapy include biological natural products. An increasing interest has been reported on the use of biologically active substances from food (Cui *et al*, 2013; Comstock *et al*, 2014). Milk and dairy products have become recognized as functional foods, suggesting their use has a direct and measurable effect on health outcomes, namely that their consumption has been related with a reduced risk of numerous cancers (Marshall, 2004; Esmat *et al*, 2013).

In vivo studies showed that oral administration of bovine LF to rodents significantly reduces chemically induced tumorigenesis in different organs (breast, esophagus, tongue, lung, liver, colon and bladder) and inhibits angiogenesis (Tsuda *et al*, 2002; Iigo *et al*, 2009). It has been demonstrated that more than 60% of administrated bovine LF survived passage through the adult human stomach and entered the small intestine in an intact form (Troost *et al*, 2001). Intact and partly intact bovine LF are likely to exert various physiological effects in the digestive tract. Moreover, subcutaneously

administration of LF inhibited the growth of implanted solid tumors and exerted preventive effects on metastasis (Bezault *et al*, 1994). These activities of LF have been attributed to its immunomodulatory potential and ability to activate T and NK cells (Damiens *et al*, 1999). Furthermore, LF was found to induce apoptosis in several human cell lines, as for example A459 lung cells, CaCO-2 intestine cells and HTB9 kidney cells (Hakansson *et al*, 1995). Moreover, LF was effective against melanoma cells (Pan *et al*, 2007), head and neck cancer cells (Xiao *et al*, 2004).

There is no study about the effect of goat milk Lactoferrin on any cancer cell line in Iraq. Therefore, this project was designed to study the effect of a range of LF concentrations at different exposure times on cell viability by using cancer cell line as fallows:

1. Isolation and purification of Lactoferrin from goat colostrum by ion exchange chromatography and gel filtration and study some of its 2. Study the cytotoxic effect of purified goat Lactoferrin on the growth of cancer cell lines (AMN-3) and on normal cell line (REF) *in vitro*.

MATERTAILS AND METHODS

Goat colostrums

Goat colostrum was obtained from Ruminants researches station, Directorate for Agricultural Researches, Ministry of Agriculture, Abu-Grip, Baghdad. The samples were collected within the first five days after goat parturition and were immediately frozen and stored at -18°C until use.

Preparation of acid colostrum whey

The colostrum was skimmed by centrifugation in a Sigma MA3-18 centrifuge at 4000 g/min for 30 min at 4°C. Colostrum whey was prepared by precipitation of the casein from skimmed colostrum in acidic condition with gradual addition from 1N HCl until pH reached to 4.6, the precipitated casein was removed by centrifugation at 10000g/min for 15 min at 4°C. The supernatant (whey) was adjusted to pH 6.8 with 1N NaOH and dialyzed against distill water for 18 hr and then stored at –18°C until use (Al-Mashakhi and Nakai, 1987).

Isolation and purification of lactoferrin

Isolation and purification procedures by Yoshida *et al* (2000) were used to separate lactoferrin from other proteins in goat colostrums. The procedure involved cation exchange chromatography (CEC) using cation exchanger carboxymethyl Sephadex-G50 (CM-SephadexG-50) and gel filtration chromatography by using Sephadex G-200.

Cell Growth (Freshney, 2005)

Ahmed-Mohammed-Nahi- 2003 (AMN-3 cell line and fibroblastic and epithelial cells with normal chromosomal picture (REF) a normal murinecell lines were kindly provided from Iraqi center of cancer and medical genetic researches were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, Streptomycin (100 U/ml), penicillin (100 U/ml) and incubated in 5% CO_2 at 37ºC for 24 h. Cell counts determined using 0.2 ml of trypan blue solution and 1.6 ml PBS, then subculture when monolayer's cells were confluent (Cui *et al*, 2013). Afterwards, 200 µl of cells in growth medium were added to each well of a sterile 96-well microtiter plate. The plates were sealed with a self-adhesive film, lid placed on and incubated in 5% CO_2 at 37°C. When the cells are in exponential growth, *i.e.* after lag phase, the medium was removed and serial dilutions of glf extractin SFM (5000 µg/ml- 2500µg/ml, 1250µg/ml, 625µg/ml, 312.5 µg / ml, 156.25 µg /ml, 78.125 µg/ml,39.625 µg /ml and 19.53 µg/ml) were added to the wells. Four replicates were used for each concentration of Lactoferrin. The middle two columns as control (cells treated with SFM only). Afterwards, the plates re-incubated under the same condition for the selected exposure times (24, 48, 72 hrs).

Cytotoxicity assay

200µl of cell suspension (Confluent monolayer's) of both AMN3 and REF were seeded into wells of a 96-well plate. After 24 hrs of incubation 200 µl of glf extract serial dilutions were added. Four replicates were used for each concentration of extract. Afterwards, the plates were re-incubated at 37°C for the selected exposure times (24, 48, 72 hrs). The cytotoxicity test was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Esmat *et al*, 2013). In brief, 50 µl of MTT was added to the wells, the cells were cultured for additional 4 hrs at 37ºC. Then 100 µl of DMSO was added to the wells. The solubilized formazan was measured at 550nm using microplate spectrophotometer (Multiskan, Finland).

The % inhibition were calculated with the following formulae :

Inhibition % = 1- (OD of sample / OD of control) × 100.

RESULTS

Isolation and purification of lactoferrin

Two steps were used to purify lactoferrin from colostrum whey, ion exchange chromatography and gel filtration were applied, respectively.

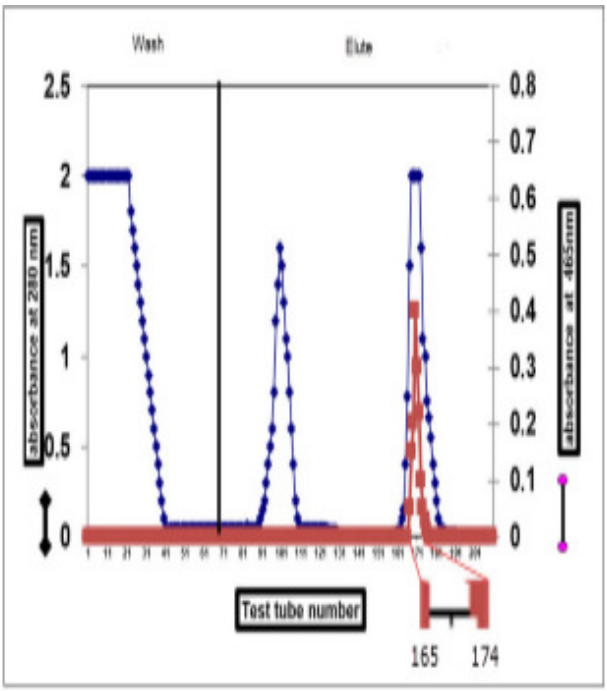

Fig. 1 : Ion exchange chromatography for purification gLF by using CM-Sephadex G-50 (2×24 cm) column, equilibrated with Tris-HCL buffer (0.05M, pH7.5), eluted with Tris-HCL buffer with NaCl 0.2M and 0.5 M in flow rate 18 ml/hr., 3ml for each fraction.

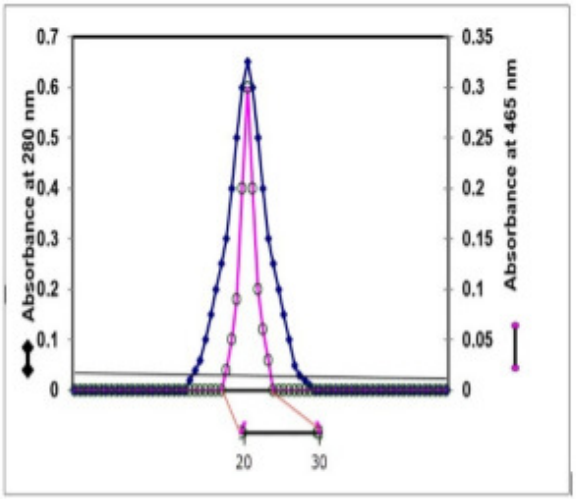

Fig. 2 : Gel filtration chromatography for purification gLF by using Sephadex G-200 column (1.5 × 60 cm), equilibrated with 0.5 M phosphate buffer containing 0.01 M NaCL, pH 7.4 with a flow rate of 18 ml/hr, 3ml for each fraction.

Ion-exchange chromatography

CM-Sephadex G-50 cation exchanger was used in goat milk lactoferrin purification due to its important properties such as easy of preparation and the possibility of reused after reactivated (Bonner, 2007). Whey, which prepared in above paragraph was pass through CM-Sephadex G-50 column, 0.05M of Tris HCl buffer pH 7.5 solution was used as equilibration buffer. Fig. 1 shows

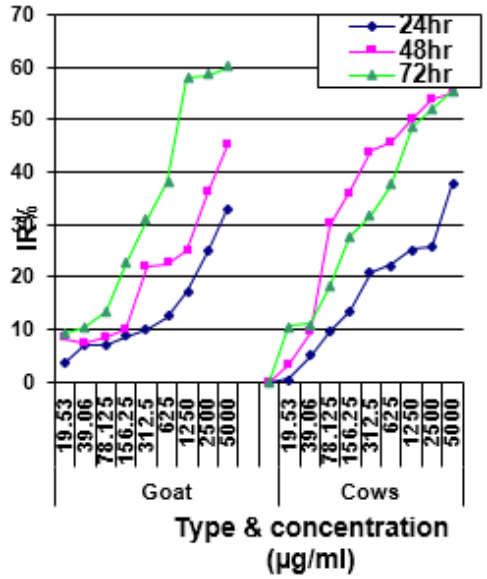

Fig. 3 : The inhibitory effects of gLF and bLF on AMN-3 cell line growth during different periods of exposure.

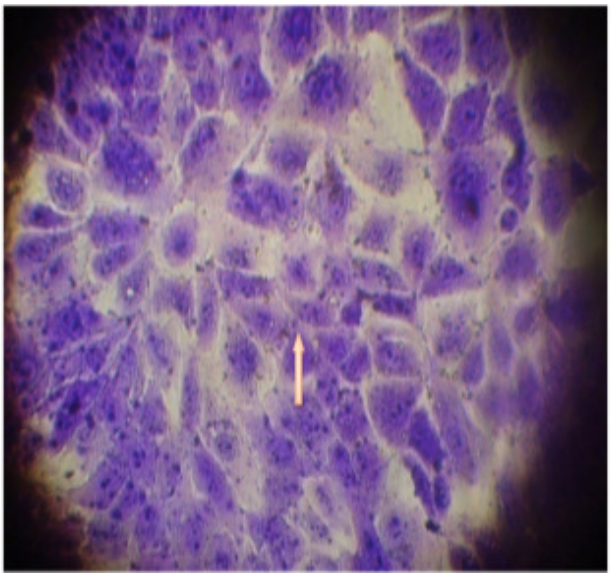

Fig. 4 : Confluent monolayer (↑) in control group of AMN-3 cell line with wall differentiation, no empty spaces (X100), (crystal violet stain).

one protein peak appeared in the washing step belong to unbounded proteins, while two protein peaks appeared in the elution the first protein peak with green color refers to the lactoperoxidase enzyme, while the second protein peak was given pink color, which shows high value when readon the wave length of 465nm Special detects for protein lactoferrin (Shimazaki *et al*, 1992). Results indicated that the second peak, which has LF that appeared in fraction (165-175) and eluted by using 0.5M of NaCl. The fractions of second peak were pooled and desalted by dialyzing against distilled water overnight and then concentrated by sucrose.

Table 1 : Mean values of inhibition rate percentage (IR %) of AMN3 cell line after treatment with different concentrations of gLF for 24, 48 &72 hours.

Concentration (µg/ml)	IR%			LSD value
	24hr	48hr	72hr	
19.53	31.20	29.31	43.41	7.42 *
39.06	36.52	34.01	36.22	5.34NS
78.125	37.94	37.39	41.31	5.81NS
156.25	39.36	40.13	42.00	4.79NS
312.50	40.42	45.65	47.07	5.31 *
625	41.48	49.56	50.12	7.92 *
1250	43.61	50.43	50.23	6.97NS
2500	49.29	50.43	51.03	5.23 *
5000	51.41	56.52	56.14	6.74 *
LSD value	8.92*	8.57*	7.13*	---
* (P<0.05), NS: Non-significant.				

Table 2 : Mean values of inhibition rate percentage (IR %) of AMN-3 cell line after treatment with different concentrations of goat and cow milk LF for 24, 48 & 72 hours.

Concentration (µg/ml)	Goats IR%			LSD value	Cows IR%			LSD value
	24hr	48hr	72hr		24hr	48hr	72hr	
19.53	31.20	29.31	43.41	7.42 *	30.14	20.95	27.13	6.31 *
39.06	36.52	34.01	36.22	5.34NS	32.97	26.30	32.40	6.84NS
78.125	37.94	37.39	41.31	5.81NS	32.97	30.14	35.17	5.98NS
156.25	39.36	40.13	42.00	4.79NS	32.97	34.32	36.01	5.38NS
312.50	40.42	45.65	47.07	5.31 *	33.33	35.71	40.31	5.09 *
625	41.48	49.56	50.12	7.92 *	33.33	30.86	42.71	5.62 *
1250	43.61	50.43	50.23	6.97NS	37.58	41.30	48.23	6.02 *
2500	49.29	50.43	51.03	5.23 *	40.42	50.43	53.15	5.84 *
5000	51.41	56.52	56.14	6.74 *	56.02	56.95	55.27	5.24NS
LSD value	8.92*	8.57*	7.13*	---	8.05*	8.64*	9.13*	---
* (P<0.05), NS: Non-significant.								

The results of this study compatible with several other studies, Nam *et al* (1999) purified LF from goat milk by usig CM-Toyopearl 650M column followed with AF-Heparin Toyopearl column and they found two protein peaks in elution the first peak belonged to lactoperoxidase enzyme and the second peak belonged to LF. Angeles *et al* (2008) isolated LF from Carabo's (*Bubalus bubalis* L.) milk whey by ammonium sulfate precipitation and cation exchange chromatography using carboxymethyl cellulose (CMC). Younghoon *et al* (2009) purified LF from goat colostrum by CM-Sephadex G-50 ion-exchange column and affinity chromatography on *Hi-Trap* Heparin HP. Moradian (2014) enabled purified LF from colostrum of cow's milk with one step by using CM-sephadex G-50, a cation exchange chromatography and they determined LF concentration by Bradford assay, which was about 2.4 mg/ml, with good biological activity and purification efficiency was about 90%.

Gel filtration chromatography

After purification by ion exchange, fractions representing LF were collected and dialyzed against distilled water then concentrated by sucrose for applying to gelfiltration chromatography by using Sephadex G-200 column. Results in Fig. 2 showed one protein peak with light pink color appeared in the eluted fractions (20 to 30) had the LF protein, which shown high value when readon the wave length of 465nm Special detects for protein Lactoferrin. Gel filtration method was used widely in extra.

Legrand *et al* (2008) purified LF from human milk by Sephadex G-200 column, while Kim *et al* (2009) applied Sephadex G-100 column in purification of LF from mare milk.

Cytotoxic activity of lactoferrin on several cell lines

Growth inhibitory effect

The result of the cytotoxic activity of glf tested against Ahmed-Mohammed-Nahi- 2003 (AMN-3) determined by MTT assay and percentage of inhibition calculated by microplate reader at 550nm were listed in Table 1.

In 24h, 48 and 72h incubation with sample varying concentration, the extract has shown remarkable anticancer activity. The glf extract had induce cell death in a confluent cancer cell population to a varying percentage according to their varying concentration. For instance 33.12% cell were died at the concentration of 5000µg/ml after 24h exposure, whereas 60.11% cell died at the same concentration after 72h incubation, these results revealed time–dependent response. The results shows that a meaningful of non linear regression of (Logarithmic) model for the statistical hypothesis between the two factors % cell inhibition at 24h, 48h and 72h times and concentration. The slop value at % inhibition 24h indicating that with increasing of concentration, a meaningful changeability should be occurred in the inhibition with logarithmic regression equation and that estimate a highly significant effect at P<0.01, as well as, strong correlation coefficient had been reported between the studied factors with highly significant at P<0.01. Also the long term trend between the two factors, % Cell inhibition and concentration, which indicating that a highly responding are accounted with % cell inhibition up to 50 µg/ml (conc.). Furthermore, the results of % Cell inhibition at 72h. time of exposure and concentration, shows that in increasing of concentration scale, a meaningful changeability should be occurred in the % cell inhibition (72h.) estimate a significant effect at P<0.05. The results obtained from treating REF cell line with glf extract are

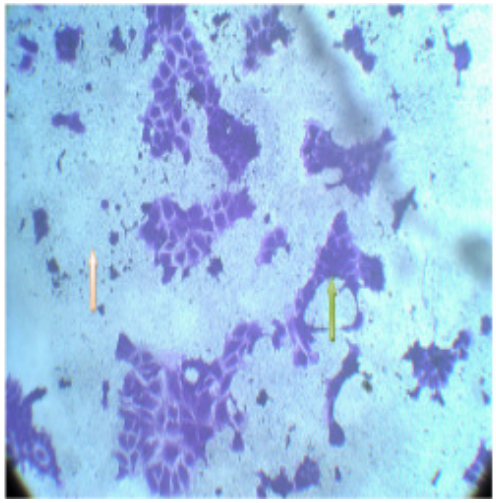

Fig. 5 : AMN-3 cell line reveals great loss of cellular features ( ) and large number of dead cells ( ) after exposure to bLF for 72 hr, 100X, crystal violet.

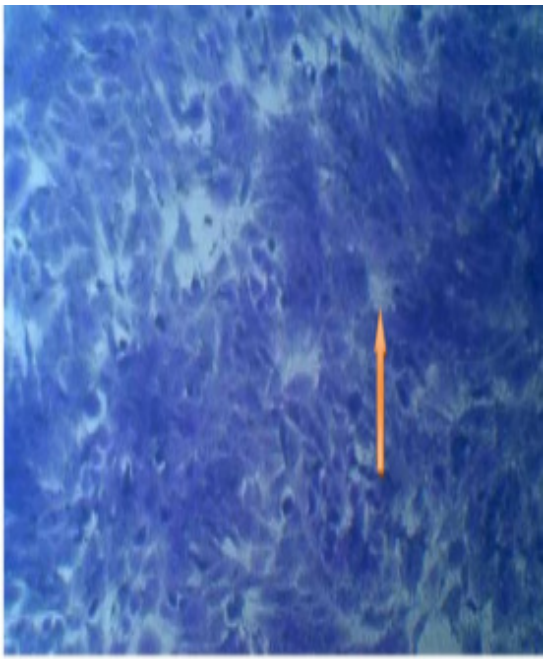

Fig. 6 : REF cell line shows confluent monolayer ( ) no empty spaces, (control) 40X, crystal violet.

presented in Table 2, which showed decreasing in growth of REF cell as compare to control culture.

In 24h exposure the lower concentration 19.53 µg/ml showed no cytotoxicity, while slightly inhibition rate 5% at the same concentration after 72h exposure time.

Inhibition percentage was slowly increased with increasing the concentration (6.34%, 10.45%, 10.59, 10.72%, 11.08%, 11.14%, 17.28%, 17.59%, 19.22%) (19.53, 39.06, 78.125, 156.25, 312.50, 625, 1250, 2500, 5000) and long exposure time respectively, despite of no significant difference at P = 0.075.

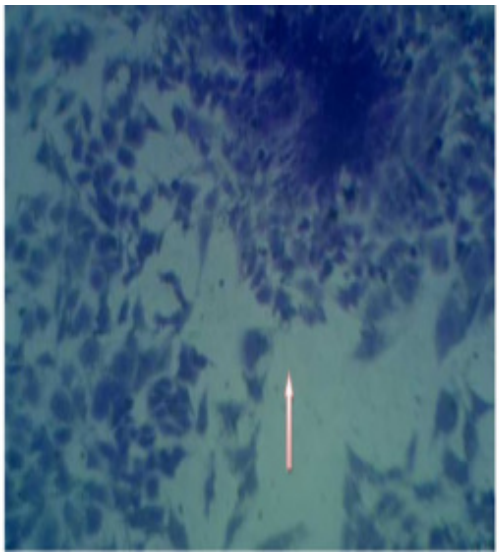

Fig. 7 : REF cell line shows space between cells ( ) after exposure to gLF for 72 hr. 40X, crystal violet.

Table 3 : Cytotoxicity of gLf on REF after 72h exposure.

Concentration (µg/ml)	IR% 72 hr
19.53	6.34
39.06	10.45
78.125	10.59
156.25	10.72
312.50	11.08
625	11.14
1250	17.28
2500	17.59
5000	19.22

* (P<0.05).

DISCUSION

Chemoprevention and chemotherapy with naturally occurring compounds such as LF have become increasingly important strategies in inhibiting carcinogenesis and tumor growth (Fraumeni *et al*, 2006).

The effect of different concentrations of purified goat milk Lactoferrin (gLF) from (19.53 to 5000 µg/ml) on Ahmed-Mohammed-Nahi- 2003cancer cell line (AMN-3) after (24, 48 & 72) hrs of exposure was studied and the results were shown in Table 1 and Figs. 1, 2 and 3.

The results revealed significant cytotoxic effect at levels (P<0.05) for all concentrations and for all exposure time of gLF on AMN-3 cell line as compared to untreated control cells as estimated by comparison of theoptical density of the treated and control cell lines. The inhibition rate IR% increased with raising of gLF concentration and incubation period. The highest inhibitory growth was at the concentration (5000 µg/ml) after 72 hrs of exposure time. IR% values after 24h of exposure to gLF at

concentration dependent (19.53, 39.06, 78.12, 156.25, 312.50, 625.00, 1250.00, 2500.00,5000 µg/ml) were (31.20, 36.52, 37.94, 39.36, 40.42, 41.48, 43.61, 49.29 and 51.41%), respectively, while after 48 hrs the result increased and IR% values became (29.31, 34.01, 37.39, 40.13, 45.65, 49.56, 50.43, 50.43 and 56.52%), respectively. However, after 72hrs of exposure the inhibition rates increased to 43.41, 36.22, 41.31, 42.00, 47.07, 50.12, 50.23, 51.03 and 56.14%, respectively. To compare the anticancer activity between goat and cow milk LF on AMN3 tumor cell lines. Table 2 shows the effect of different concentrations of purified gLF and standard bLF after (24, 48 & 72 hrs) of exposure time on AMN3 cell line growth.

The results revealed that the bLF also possess inhibition effect on the growth of AMN3 tumor cells line and this effect appears clearly with the increase in the time of exposure, as well as with the rise in the bLF concentration. The inhibition rates after 24hrs of exposure to bLF were (30.14, 32.97, 32.97, 32.97, 33.33, 33.33, 37.58, 40.42 and 56.02%), respectively.

When the exposure time increased to 48 hrs, the inhibition rates for these concentrations reached (20.95, 26.30, 30.14, 34.32, 35.71, 30.86, 41.30, 50.43 and 56.95%), while the IR % after 72 hrs were the highest (27.13, 32.40, 35.17, 36.01, 40.31, 42.71, 48.23, 53.15 and 55.27%), respectively.

The results in Table 2, also indicated that there are significant differences between the two kinds of LF in the effect on the growth of RD tumor cell line, gLF possess a highly growth inhibition than bLF, which appeared clearly from the first concentration (19.53 µg/ml) after 72 hrs of exposure the gLF had IR% nine times more than those for bLF (9.35 and 10.65%). The differences in IR% reduced in high concentrations reached to more than three fold at concentration 156.25 µg/ml (22.58 & 27, 55%) and two fold at 312.50 µg/ml until 1250 µg/ml (31.12 & 21.35%), while at the high concentration 5000 µg/ml after 72hr of exposure time IR% values reach to 60.11 and 55.48% for gLF and bLF, respectively. There are ten suggested mechanisms underlying chemopreventive potential for any material, including the antioxidant, anti-inflammatory, immune-enhancing, anti-hormone effects, modification of phase-1 drug-metabolizing enzymes, oncogene modification, regulation of cell growth, regulation of cell differentiation, promotion of apoptosis and inhibition of angiogenesis (Mulder and Morris, 2010). Tsuda *et al* (2004) reported that LF possesses immune-modulating, antioxidant and anti-inflammatory properties which together support its anticancer activity. The previous studies have found that down-regulation of the LF gene could be associated with higher incidence of breast cancers (Furmanski *et al*, 1989). On the other hand, the exogenous supply of LF and its variants were reported to efficiently inhibit the cancer growth both *in vitro* and *in vivo* (Yamada *et al*, 2008; Xu *et al*, 2010; Kanwar and Kanwar, 2013; Park *et al*, 2013). The results of this study were consistent with those of other studies. Younghoon *et al* (2009) studied the anticancer activity of goat and bovine milk LF and they found that the two kinds of LF inhibit the growth of certain experimental cancer cell line, human colorectal cancer cell line HT-29, human uterus cancer cell line RD, human lung cancer cell line A549, human gastric cancer cell line KATO-111 and human breast cancer cell line ZR-75-1, but the bLF was more effective than gLF, the anticancer effect of LF is suggested to be mediated via two different routes by directly affecting tumor cell growth and through NK-cell activation. The iron-binding properties also contribute to the anticancer properties of LF, since free iron may act as a mutagenic promoter by inducing oxidative damage to nucleic acid structure (Weinberg, 2004). Also, Nandhini and Palaniswamy (2013) studied the anticancer effect of goat milk fermented by *Lactobacillus plantarum* and *Lactobacillus paracasei* using HeLa cell line and the cell viability was assayed by MTT they observed that the cell viability decreased with the increased in the concentration of gLF hydrolysate.

REFRENCES

Al-Mashikhi S A and Nakai S (1987) Isolation of bovine immunoglobulins and lactoferrin from whey protein by gel filtrations techniques. *J of Dairy Science* **70**, 2486-2492.

American Association for Cancer Research (2013) AACR Cancer Progress Report 2013. *Clin. Cancer Res.* **19**(Supplement 1), S1-S88.

Bezault J, Bhimani R, Wiprovnick J and Furmanski P (1994) Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. *Cancer Research* **54**, 2310-2312.

Comstock S S, Reznikov E A, Contractor N and Donovan S M (2014) Dietary bovine Lactoferrin alters mucosal and systemic immune cell responses in neonatal piglets. *J Nutr*. **4**, 525-532.

Cui M Y, Jack H W, Jiang X and Tzi B N (2013) Studies on anticancer activities of Lactoferrin and Lactoferricin. *Current protein and peptide Science* **14**, 492-503.

Damiens E, El-Yazidi I, Mazurier J, Duthillie I, Spik G and Boilly-Marer Y (1999) Lactoferrin inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma cells. *J Cell Biochem.* **74**, 486–498.

Esmat A, Gaspar R and Carmen F (2013) Structure and Functions of Lactoferrin as Ingredient in Infant Formulas. *J of Food Res*. **2**, 25-36.

Ferguson L (2009) Nutrigenomics approaches to functional foods. *J.*

Am. Diet. Assoc. **109**, 452-458.

Freshney R I (2005) *Culture of animal cells: A Manual for basic asic technique* (**5**th ed.). John Wiley and Sons Inc. Publication,New York.

Fraumeni J F, Robert J, Leo S D and Kinlen J (2006) Cancer. *Principles and Practice of Oncology*. **3**rd ed. p 13-16.

Furmanski P, Li Z, Fortuna M B, Swamy C and Das M R (1989) Multiple molecular forms of human lactoferrin. Identification of a class of lactoferrins that possess ribonuclease activity and lack iron-binding capacity. *Journal of Experimental Medicine* **170**, 415e429.

Hakansson A, Zhivotovski B, Orrenius S, Sabharwal H and Vanbory C S (1995) Apoptosis induced by a human milk protein. *Proc. Natl. Ac. Sci.* **92**, 8064-8068.

Hatzimichael E and Crook T (2013) Cancer Epigenetics: New Therapies and New Challenges. *Journal of Drug Delivery* Vol. 2013, Article ID 529312, 9 pages.

IARC (International Agency for Research on Cancer) (2011) Section of Cancer Information. Cited by: Yousif R A (2012). Effect of *Cordia myxa* L. crude extract on growth of cancer and normal celllines. *M. Sc. Thesis.* College of Science for Women, University of Baghdad , Iraq.

Iigo M, Alexander D B, Long N, Xu J, Ukamachi K, Futakuchi M, Takase M and Tsuda H (2009) Anticarcinogenesis pathways activated by bovine Lactoferrin in the murine small intestine. *Bio-Chimi*. **91**(1), 86-101.

Jacobs D, Gross M and Tapsell L (2009) Food synergy: an operational concept for understanding nutrition. *Am. J. Clin. Nutr*. **89**, 1543-1554.

Kanwar R K and Kanwar J R (2013) Immunomodulatory lactoferrin in the regulation of apoptosis modulatory proteins in cancer. *Protein Pept Lett.* **20**(4), 450-458.

Marshall K (2004) Therapeutic applications of whey protein. *Alt. Med Rev*. **9**(2), 136-156.

McCabe-Sellers B, Chenard C, Lovera D, Champagne C, Bogle M and Kaput J (2009) Readiness of food composition databases and food component analysis systems for nutrigenomics. *J. Food Comp. Anal*. **22**, S57-S62.

Mulder A M and Morris C A (2010) Lactoferrin in Immune Function, Cancer and Disease Resistance. Dietary Components and Immune Function. Chapter 17 / Humana press Springer New York.

Nandhini B and Palaniswamy M (2013) Anticancer effect of goat milk fermented by *Lactobacillus plantarum* and *Lactobacillus paracasei*. *Int J of Pharmacy and Pharmaceutical Sci*. **5**, 898-901.

Pan Y, Rowney M, Guo P and Hobman P (2007) Biological properties of Lactoferrin: an overview. *Aust. J. Dairy Technol*. **62**, 31-42.

Park Y G, Jeong J K, Lee J H, Lee Y J, Seol J W, Kim S J, Hur T Y, Jung Y H, Kang S J and Park S Y (2013) Lactoferrin protects against prion protein-induced cell death in neuronal cells by preventing mitochondrial dysfunction. *Int. J. Mol Med*. **31**, 325–330.

Rastogi T, Hildesheim A and Sinha R (2004) Opportunities for cancer epidemiology in developing countries. *Nature Rev. cancer* **4**, 909.

Ronald R W, Sherma Z and Vector R B (2010) Dietary Components and Immune Function. Humana press Springer New York Dordrecht Heidelberg London Library of Congress Control Number: 2010931836.

Siegel R, Naishadham D and Jemal A (2012) Cancer statistics, 2012. *CA: A Cancer Journal for Clinicians* **62**, 10–29.

Troost F, Steijns J, Saris W and Bruumer R (2001) Gastric digestion of bovine lactoferrin in vivo in adults. *J Nutr.* **131**, 2101–2104.

Tsuda H, Sekine K, Fujita K and Ligo M (2002) Cancer prevention by bovine lactoferrin and underlying mechanisms – a review of experimental and clinical studies. *Biochem Cell Biol.* **80**, 131–136.

Tsuda H, Ohshima Y, Nomoto H, Fujita K, Matsuda E, Iigo M, Takasuka N and Moore M (2004) Cancer prevention by natural compounds. *Drug Metab Pharmacokinet* **19**, 245–263.

Weinberg E D (2004) Suppression of bacterial biofil formation by iron limitation. *Med Hypotheses* **63**, 863-865.

Xiao Y, Monitto C, Minhas K and Sidransky D (2004) Lactoferrin down-regulates G1 cyclin-dependent kinases during growth arrest of head and neck cancer cells. *Clin Cancer Res.* **10**, 8683–8686.

Xu X, Jiang H, Li H, Zhang T, Zhou Q and Liu N (2010) Apoptosis of Stomach Cancer Cell SGC- 7901 and Regulation of Akt Signaling Way Induced by Bovine Lactoferrin. *Journal of Dairy Science* **93**(6), 2344-2350.

Yamada Y, Sato R, Kobayashi S, Hankanga C, Inanami O and Kuwabara M (2008) The antiproliferative effect of bovine lactoferrin on canine mammary gland tumor cells. *Journal of Veterinary Medical Science/Japanese Society of Veterinary Science* **70**, 443.

Yoshida A, Wei Z, Shinmura Y and Fukunaga N (2000) Separation of Lactoferrin-a and -b from bovine colostrum. *J. Dairy Sci.* **83**, 2211-2215.

Younghoon K, Mae J, Kyoung S H, Jee Y I, Sejong O and SAE H K (2009) Anticancer activity of Lactoferrin isolated from caprine colostrum on human cancer cell lines. *Intern Journal of Dairy Techn*. **62**, 277–281.