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Study The Effect of Purified *Goat* Milk Lactoferrin on HeLa Cancer Cell line Growth *In vitro*

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Abstract:

Lactoferrin is an important protein in many biological applications as a potential cancer treatment agent .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 evaluted 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, HeLa (Human cervical cancer) 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 HeLa 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 72hrs of exposure time (64.38%) ,while only the highest concentration gave significant inhibitory effect ($P< 0.05$) with normal cell REF .

Keywords: *goat lactoferrin, HeLa, 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(1).

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(2) . The world health organization (WHO) estimated that if unchecked, annual global cancer deaths could rise to 15 million by 2020(3) . Recently in Iraq there is a terrible number of unpublished cancer cases beside the published cases by the Iraqi Cancer Council in 2008 were 19.9 thousand and more than 15.4 thousand of them were died(4). The current conventional cancer treatment options for localized tumors and advanced disease are typically associated with risks and side effects (5) .The discovery of anticancer drugs remains a highly challenging endeavor and cancer a hard-to-cure disease(6) .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(7)(8).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(9)(10).

Invivo 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(11)(12)).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(13) .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(14).These activities of LF have been attributed to its immunomodulatory potential and ability to activate T and NK cells (15)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(16).Moreover, LF was effective against melanoma cells (17)head and neck cancer cells (18).

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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 characters, (Molecular weight, Carbohydrate content and Iron content).

2-Study the cytotoxic effect of purified goat Lactoferrin on the growth of cancer cell lines (HeLa), and on normal cell line (REF) in vitro.

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. (19)

Isolation and Purification of lactoferrin

Isolation and purification procedures by (20)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-Sephadex G-50) and gel filtration chromatography by using Sephadex G-200

Cell Growth(21)

Human cervical cancer cell line (HeLa) and fibroblastic and epithelial cells with normal chromosomal picture (REF) a normal murine cell lines were kindly provided from experimented therapy department cell bank unite 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% CO2 at 37 ºC 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. 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% CO2 at 37°C. When the cells are in exponential growth, i.e. after lag phase, the medium was removed and serial dilutions of glf extract in SFM (5000µg/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 ethanolic extract. 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

Two hundred µl of cell suspensions (1x105 cell/ml) (Confluent monolayer's) of both Hela 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. 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 550 nm using microplate spectrophotometer (Multiskan, Finland).

The % Inhibition were calculated with the following formulae :

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

Results:

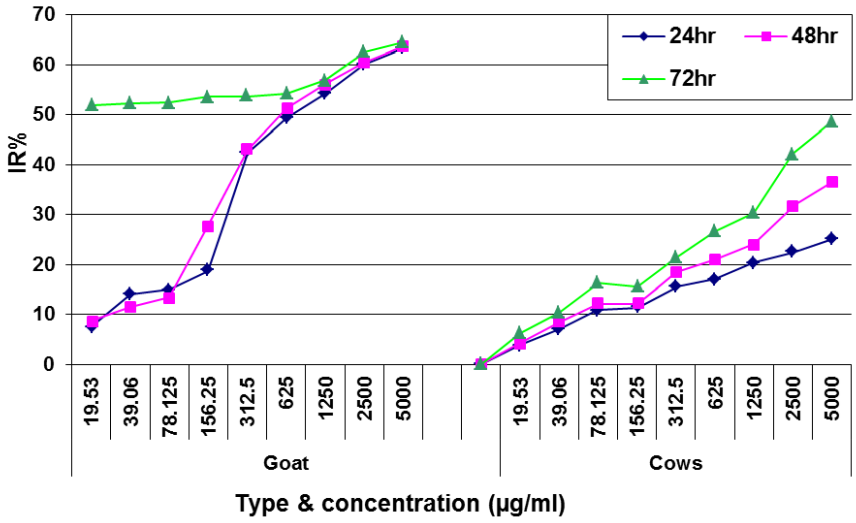
The result of the cytotoxic activity of glf tested against Human cervical cancer cell line (HeLa) determined by MTT assay and percentage of inhibition calculated by microplate reader at 550nm , were listed in table (1).

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

Lactoferrin concentration (µg/ml)	IR%			LSD value
	24hrs	48hrs	72hrs	
19.53	7.58	8.66	51.94	8.37*
39.06	13.93	11.48	52.19	8.09*
78.12	14.93	13.48	52.49	9.53*
156.25	18.72	27.74	53.50	8.57*
312.50	42.31	42.93	53.61	9.02*
625	49.29	51.23	54.08	8.22*
1250	54.27	56.14	56.84	9.59*
2500	59.98	60.34	62.45	7.41*
5000	63.35	63.67	64.38	7.29*
LSD value	10.604*	11.739 *	8.364 *	---
* (P<0.05), NS: Non-significant.				

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

LF concentration (µg/ml)	Goats			LSD value	Cows			LSD value
	IR%				IR%			
	24hr	48hr	72hr		24hr	48hr	72hr	
19.530	7.58	8.66	51.94	8.37*	3.90	4.21	6.25	4.83NS
39.060	13.93	11.48	52.19	8.09*	7.03	8.42	10.33	4.97NS
78.125	14.93	13.43	52.45	9.53*	10.78	12.10	15.50	4.38 *
156.250	18.72	27.74	53.50	8.57*	11.35	12.10	16.25	6.74NS
312.500	42.31	42.93	53.61	9.02*	15.61	18.42	21.35	6.92NS
625	49.29	51.23	54.08	8.22*	17.02	20.91	26.60	5.68 *
1250	54.27	56.14	56.83	9.59*	20.35	24.05	30.21	6.21 *
2500	59.98	60.34	62.45	7.41*	22.41	31.57	42.11	6.53 *
5000	63.35	63.67	64.38	7.29*	25.00	36.57	48.66	7.91 *
LSD value	10.604 *	11.739 *	8.364 *	---	7.02*	8.64*	8.92*	---
* (P<0.05), NS: Non-significant.								



Figure(1): The inhibitory effects of gLF and bLF on HeLa cell line growth during different periods of exposure.

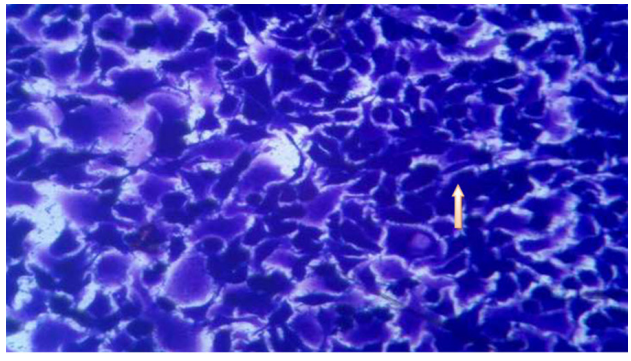


Figure (4. 8): HeLa cell line shows confluent monolayer ( ), cohesive malignant cell control 100X, crystal violet.

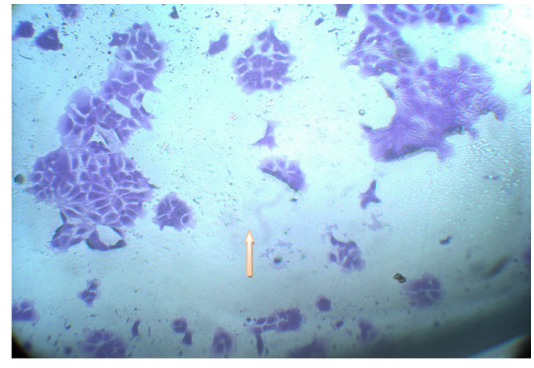


Figure (4-9): HeLa cell line shows highly reduce in viability cell number ( ) after treatment with gLF at 72 hr., 100X, crystal violet.

In 24h,48 and 72h incubation with sample varying concentration, The glf extract had induce cell death in a confluent cancer cell population to a varying percentage according to their varying concentration .For instance 63.35% cell were died at the concentration of 5000µg/ml after 24h exposure, whereas 64.38% 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 presented in table (2) which showed decreasing in growth of REF cell as compare to control culture.

Table (2): cytotoxicity of gLf on REF after 72h exposure.

Concentration (µg/ml)	IR% 72h
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).**

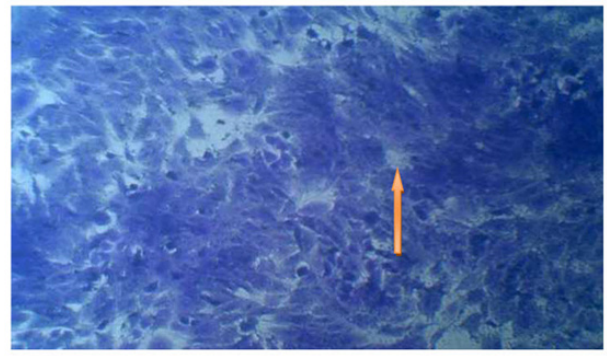


Figure (4.19): REF cell line shows confluent monolayer ( ), no empty spaces, (control) 40X, crystal violet.

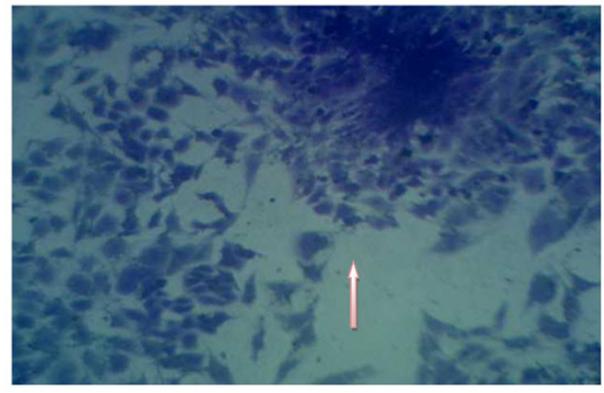


Figure (4.20): REF cell line shows space between cells ( ) after exposure to gLF for 72hr. 40X, crystal violet.

In 24h exposure the lower concentration 19.53µg/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).

Discussion:

The effect of different concentrations of purified goat milk Lactoferrin (gLF) from (19.53 to 5000 µg/ml) on human cervical cancer cell line (HeLa) after (24, 48 &72) hrs of exposure was studied and the results were shown in Table (1) and Figures (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 HeLa cell line as compared to untreated control cells as estimated by comparison of the. optical 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 72hrs of exposure time .IR% values after 24h of ex-

posure 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 (7.582, 13.928, 14.928, 18.720 ,42.310 ,49.289, 54.265, 59.981 and 63.350 %) respectively ,while after 48 hrs the result increased and IR% values became (8.65, 11.48, 13.42, 27.74,42.93,51.23,56.14,60.43 and 63.67%) respectively. However after 72hrs of exposure the inhibition rates increased to (51.94 ,52.19, 52.49, 53.50, 53.61, 54.08, 56.84, 62.45 and 64.32 %) respectively. To compare the anticancer activity between goat and cow milk LF on HeLa tumor cell lines Table (2) shown the effect of different concentrations of purified gLF and standard bLF after (24, 48 &72) hrs of exposure time on HeLa cell line growth .

The results revealed that the bLF also possess inhibition effect on the growth of HeLa 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 (3.90,7.03,10.78,11. 35,15.61,17.02,20.35,22.41and 25.00%) respectively

When the exposure time increased to 48 hrs, the inhibition rates for

these concentrations reached (4.21,8.42, 12.10, 12.10, 18.42, 20.91, 24.05,31.57 and 36.57%) respectively, while the IR % after 72 hrs were the highest (6.25,10.33,15.50,16.25,21 .35,26.60,30.21,42.11 and 48.66%) 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 HeLa 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 (51.94 &6.25%). The differences in IR% reduced in high concentrations reached to more than threefold at concentration 156.25 µg/ml (53.50 &15.50%) and twofold at 312.50 µg/ml until 1250 µg/ml (53.61 & 21.35%),while at the high concentration 5000 µg/ml after 72hr of exposure time IR% values reach to 64.38 and 48.66 % 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 (22)(23) 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 (24) .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 (25)(26)(27)(28) .The results of this study were consistent with those of other studies (29) 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 HeLa, 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(30).also (31) 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.

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دراسة التاثير الخلوي السمي للاكتوفيرين المنقى من حليب الماعز في الخلايا السرطانية

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3 المركز العراقي لبحوث السرطان والوراثة الطبية/ الجامعة المستنصرية

الخلاصه:

يعد اللاكتوفيرين من المواد البروتينية ذات التطبيقات البيولوجية المتعددة والمهمة لاسيما دوره الفعال في علاج العديد من انواع السرطان . في الدراسة الحالية نقي بروتين اللاكتوفيرين من لبأ الماعز بطريقة التبادل الأيوني باستخدام عمودCM-Sephadex G-150 ثم الترشيح الهلامي باستخدام عمود Sephadex G-200 . تم التحري عن التأثير السمي الخلوي لبروتين اللاكتوفيرين المنقى من لبأ الماعز (goat milk Lactoferrin (gLF في نوعين من خطوط الخلايا هي خط خلايا سرطان عنق الرحم البشري(HeLa وخط الخلايا الليفية الطبيعي لأجنة الفار (REF) باستخدام تراكيز مختلفة منه تراوحت (53، 19 - 5000) ميكروغرام / مل ولفترات تعرض مختلفة (48، 24 و 72) ساعة. عدا خط الخلايا الطبيعية REF فدرس تأثيره عليها بعد مرور 72ساعة فقط ، تم تقييم تأثير gLF بحساب نسبة تثبيط نمو خلايا HeLa و REF من خلال الفحص باستعمال صبغة MTT أظهرت النتائج فعل gLf المضادة للسرطان يزداد بزيادة التركيز ووقت التعرض وان اعلى نسبة تثبيط بلغت 64.38 %كانت عند تركيز 5000 ميكروغرام / مل بعد فترة تعرض72 ساعة ، في حين كان تاثير gLf محدود على الخلايا الطبيعية REF الا في التراكيز العالية منه.