Cytotoxic effect of the crude extracts of the locally Isolate *Lactococcus lactis* on AMN-3 tumor cell line

التأثير السمي للمستخلصات الخام للعزلة المحلية *Lactococcus lactis* على الخط الخلوي السرطاني AMN-3

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Abstract

This project was conducted to study the activity of secondary metabolites produced by one of a human microflora which is *Lactococcus lactis* on AMN-3 tumour cell line *in vitro*. Twenty samples of dairy products (bucolic sour yoghurts, pasteurized milk and raw milk) were collected; nine isolates of *Lactococccus* were isolated by propagating in MRS broth medium followed by subjecting the isolates to microscopic, cultural, physiological and biochemical tests. The results showed the presence of four isolates belonged to the genus *Lactococcus* sp. *lactis*. the isolates were grown in in three broth production media, which were MRS, VVM and BHI at 37ºC for 6 h. followed by extraction of crude extracts using ammonium sulphate saturation ratio which was 80% for each medium confidentially. The overall result was three crude extracts (one from each medium), followed by estimation the concentrations of extracted proteins depending on standard curve of bovine serum albumin. The cytotoxic activity of different concentrations for each crude extract was studied on AMN-3 tumour cell line for three incubation periods (24,48,72) hrs in addition to normal rabbit embryo fibroblast (REF) cell line for 72 hr only. The result illustrated a clear cytotoxic activity of these crude extracts with high significances on this tumour cell line during the three incubation periods, suggesting that the cytotoxic effect of these crude extracts is a dose and time dependent, but on REF cell line, there is no significant effect of these crude extracts was reported, suggesting also, that may be the active compound of *L. lactis* possess some specificity in cytotoxicity on cancer cells but not on normal cells.

المستخلص

صُمّمت هذه الدراسة لبيان فعالية مركبات الأيض الثانوي المنتجة بواسطة أحد النبيتات الطبيعية في الجسم البشري وهي مكورات حامض اللاكتيك *Lactococcus lactis* بشكلها الخام بالاضافة الى البروتينات المستخلصة من جدارها الخلوي وتأثيرها على نمو الخلايا السرطانية خارج الجسم الحي وقد تضمنت الدراسة ما يأتي: جُمعت عشرون عينة غذائية شملت منتجات الألبان (اللبن الرائب الريفي والحليب المبستر والحليب الخام)، وعزلت منها تسع عزلات تعود للجنس *Lactococcus* تم عزلها باستخدام الوسط الزرعيMRS السائل ثم خضعت العزلات للفحوصات المجهرية والزرعية فضلا عن الاختبارات الكيموحيوية . أظهرت النتائج وجود أربع عزلات تعود للجنس *Lactococcus* النوع *lactis* .

Key Words: *L. lactis*, Antitumour activity, AMN-3 and Cytotoxicity

تم تنمية البكتيريا في ثلاثة أوساط زرعية إنتاجية سائلة وهي (VVM, MRS وBHI) عند درجة حرارة ٣٧°م لمدة ٦ ساعات ومن ثم استخلاص المستخلصات الخام باستخدام كبريتات الأمونيوم وبنسبة تشبع وهي ٨٠٪ لكل وسط على حدة. كانت المحصلة النهائية ثلاث مستخلصات خام (مستخلص واحد من كل وسط). وتقدير كميات البروتين المستخلصة بالمقارنة مع المنحني القياس لألبومين المصل البقري. بعدها تم اختبار الفعالية السمية الخلوية لتراكيز مختلفة لكل مستخلص خام من مستخلصات مكورات حامض اللاكتيك على الخط الخلوي السرطاني AMN-3 وبثلاث فترات حضن (٢٤، ٤٨، ٧٢) ساعة بالاضافة الى اختبار سمية هذه المستخلصات الخام على الخط الخلوي الطبيعي لجنين الأرنب REF ولفترة حضن واحدة فقط وهي ٧٢ساعة. كانت النتيجة وجود تأثير سمي واضح، وبمعنوية عالية لتلك المستخلصات على نمو الخلايا السرطانية وخلال فترات الحضن الثلاث، وقد لوحظ أن شدة السمية تزداد بزيادة التركيز وفترة الحضن لذا فان التأثير السمي لتلك المستخلصات الخام لمكورات حامض اللاكتيك يعتمد على التركيز وفترة الحضن، في حين لم يكن هناك تأثير واضح وذو معنوية لنفس المستخلصات في نمو الخلايا الطبيعية REF لذا قد يكون للمركبات الأيضية لمكورات حامض اللاكتيك *Lactococcus lactis* بعض التخصص في التأثير السمي على نمو الخلايا السرطانية دون الطبيعية .

Introduction

The intestinal flora is a complex ecosystem consisting of over 400 bacterial species that greatly outnumber the total number of cells making up the entire human body. These metabolically active bacteria reside close to the absorptive mucosal surface and are capable of a remarkable repertoire of transforming chemical reactions. The esophagus has a flora similar to that of the pharynx [1]. The empty stomach is sterile due to gastric acid. The normal flora of the duodenum, jejunum and upper ileum is scanty but the large intestine is very heavily colonized with bacteria among which are; Bacteriodes (mainly members of the fragilis group which outnumber *B. fragilis* itself), Bifidobacteria, Anaerobic cocci, *E. coli*, *S. faecalis*, *Clostridia*, *Lactobacilli* and less common inhabitants; *Klebsiella*spp., *Proteus* spp., *Enterobacter* spp. and *Pseudomonas aeruginosa*. The intestinal flora can be thought of as a chemical factory with massive levels of active enzymes. All rapidly growing bacterial species in the small intestines produce metabolic by-products that can be absorbed. Some of the absorbed products are utilized for energy immediately in the epithelial cells of the gut; others may be acted upon by the detoxification systems in the liver; while others are passed [2].

The enteric flora comprises approximately 95% of the total number of cells in the human body and can elicit immune responses while protecting against microbial pathogens. The beneficial role of the normal flora is the prevention of other more pathogenic bacteria from gaining a foothold in the body. The gut bacteria seem to be responsible for the normal structure and function of the intestine: they degrade mucin, epithelial cells and carbohydrate fiber and their metabolism produces vitamins, especially vitamin K [3]. However, the resident bacterialflora of the gastrointestinal tract may also be implicated inthe pathogenesis of diseases such as inflammatory bowel disease (ulcerative colitis and Crohn disease). Any compound taken orally, entering the intestine through the biliary tract or by secretion directly into the lumen is a potential substrate for bacterial transformation. So the colonic microflora is important to health [4]. Since the 1980s, many efforts have been made to better understand the molecular basis of LAB technological properties and to obtain better control of industrial processes involving LAB. This knowledge has led researchers to investigate their potential use for new applications, such as the production of heterologous proteins in bioreactors, in fermented food products or directly in the digestive tract of humans and other animals. Some LAB

used as probiotic strains, naturally exerts a positive action in lactose-intolerant consumers by providing ß-galactosidase in the gut [5]. Besides such natural benefits, another and innovative application of LAB is the antitumor activity to supplement pancreatic and gastrointestinal deficiency in humans [6].

On the strength of those investigations, the present study was proposed for isolation and identification of the species *Lactococcus lactis* that may possess antitumor activity. Extraction with partial purification of proteins produced by these bacteria in different production media in addition to their cell wall associated proteins followed by *in vitro* study of the effect of the bacterial crude extracts on the growth of AMN-3 tumor and normal cell lines.

Experimental Work

Samples Collection

Twenty samples of dairy products (bucolic sour yoghurts, pasteurized milk and raw milk) were collected in sterile containers from local markets in Baghdad governorate, followed by propagation the isolates by inoculating test tubes containing MRS broth medium with 1% of each sample and incubated at 37ºC for 24 hrs under anaerobic conditions (in an anaerobic jar).

Isolation of Lactic Acid Bacteria

Lactic acid bacteria were isolated according to Harrigan and MacCance [7], serial dilutions were performed, followed by streaking on MRS agar plates containing 1% calcium carbonate ($CaCO_3$), then incubated at 37ºC for 24 hrs. After that, a loop touch of the growth was transferred to MRS broth and preserved.

Identification of *Lactococcus lactis*

The suspected LAB isolates were identified by the following tests:

Microscopic Examination

A loop full of each isolates culture was fixed on a microscopic slide, and then stained by Gram stain to examine cell shape, Gram reaction, grouping and spore forming phenomena [8].

Biochemical Tests

The biochemical tests used to identify the locally isolate *L. lactis* are gelatinase test, catalase test, acid production and clot formation, production of ammonia from arginine, carbohydrates fermentation, growth in 4% and 6.5% NaCl, growth in different pH, growth at different temperature and growth in 0.1% methylene blue [9].

Extraction of Bacterial Secreted Proteins

Each of MRS, VVM and BHI broth were inoculated with *L. lactis* and incubated under anaerobic conditions to prevent the production of hydrogen peroxide at 37°C for 6 h to reach production phase. For extraction of protein, a cell free supernatant from each media was obtained by centrifugation at 10,000 rpm for 20 min at 4°C followed by filtration of supernatant through 0.2 µm filter unit to get cell free supernatant. The supernatant of each media was independently treated with gradual addition of solid ammonium sulphate saturation ratio 80 % [10].

Quantitative Estimation of Proteins

Protein concentration was estimated according to Bradford method by using Commassie blue G-250 and Bovine serum albumin (BSA) to determine standard curve and estimate

protein in concentrated filtrate [11].

Standard Curve of Bovine Serum Albumin

Bovine serum albumin (BSA) solution was prepared by dissolving 0.1 g of BSA in a quantity of D.W. and the volume was completed to 100 ml D.W.

Different concentrations of BSA (2, 4, 7, 10, 14, 16 and 20 µg/ml) were prepared and plotted [11].

Estimation of Extracted Proteins

The same steps followed in standard curve were used to determine the protein in concentrated filtrate by taking 20 µl from each extract.

Viable Cell Count

Cells count were performed according to Freshney [12], using try pan blue dye, dead cells take up the dye making them easily distinguished from viable cells. Which counted by Neubauer chamber.

Tumor Inhibition Assay

The procedure below was depending on cytotoxicity testing mentioned in Freshney [12], AMN-3 cell line which was applied at the passage 50 that represents mammary adenocarcinoma of female mice Balb/c that have *in vivo* spontaneous mammary adenocarcinoma. On the other hand, rabbit embryo fibroblast (REF) was used as control. Cell suspension of both cell lines were well mixed and treated with different concentrations of each extract which were (10, 5, 2.5, 1.25, 0.625, 0.312) µg / ml poured into 96 flat bottom well micro titer plate and incubated at 37 °C for (24, 48, 72) hrs in an incubator supplemented with 5% CO_2. On the other hand, the same cell line (AMN-3) cells was incubated for the same period without treating and used as control. After elapsing the incubation period, 50 µl/well of neutral red dye were added and incubated again for 2 hrs. The results were read by ELISA reader at 492 nm.

Statistical Analysis

The results were statistically analyzed to determine the significance effect among the concentrations of both extracts and their effect on tumor cell line and normal cells. The comparison between groups has based on analysis of variance test (ANOVA), while the significance differences based on Duncan's test [13].

Results and Discussion

The colonies of some isolates on MRS agar by pour plate were circular, white to yellow in color some of which were gray, smooth, mucous, bright and convex, Table (1). These characteristics were the same to those observed when pure culture was obtained using streaking method. It was observed that the growth of LAB on MRS agar was heavier than their growth on SL medium this may be due to the high ratio of sodium acetate and high acidity [14].

Also, there were other isolates their colonies characterized by different shapes (fusiform, ovoid and circular) white to pale in color, soft with smooth edges, non bright and some of which were convex. Such cultural characteristics are concerned with those of the colonies of the genus *Lactobacillus* [15].

Microscopic examination after Gram staining showed that some suspected cells were spherical, tend to be ovoid, and grouped in pairs, tetrads and short chains, Gram positive and non spore forming, Table (1), these results are supported by those of [16] when they

isolated 237 isolate of *L. lactis* among a total of 2000 isolates of LAB obtained from traditional Egyptian dairy products (different types of raw milk, ras, domiatti and kareish cheese, mish, cream, butter and fermented milk) obtained from different regions in Egypt.

Also, it was observed that the other isolates that may be belong to the genus *Lactobacillus* characterized as long curved rods, arranged in short and long chains (3-8) cells and some of which were single and in pairs, Gram positive and non spore forming.

Depending on the results of the cultural and microscopic tests, nine isolates may be belonging to the genus *Lactococcus* referred to as (Lc1, Lc2, Lc3, Lc4, Lc5, Lc6, Lc7, Lc8, Lc9) and the other tested isolates may be belonging to the genus *Lactobacillus*. The abundance of the genus *Lactococcus* in dairy and dairy products is reasonable because they possesses proteinase enzyme system encourage them to grow in milk and its products [17].

Since the present study focused on studying the effect of the crude extract of the genus *Lactococcus* on tumor cell lines, so we restricted the biochemical tests on these suspected isolates ignoring the others.

Table (1): Morphological and Cultural Taxonomic Characteristics of the Suspected Isolates.

Characteristics	Results
1- Gram stain	**G +ve**
2- Cell shape	**Spherical to oval**
3- Grouping	**Single, in pairs and short chains**
4-Colony shape on MRS and SL agar	**Circular, small, regular, convex with smooth edges**
5- Motility	**Non motile**
6- Growth under aerobic conditions	+
7- Growth under anaerobic conditions	+

+ = Presence of growth

Biochemical characteristics shown in Table (2) demonstrated that each of the nine isolates were catalase negative since no bubbles were observed after addition of hydrogen peroxide, gelatinase negative, and the isolates (Lc1, Lc4, Lc5 and Lc8) produced ammonia from arginine due to the presence of arginine deiminase which isone of three enzymes that comprise the AD system, AD catalyzes the conversion of L-arginine to citrulline,with the concomitant production of ammonia [18]. Also these four isolates have grown in 0.1% methylene blue with reduction of dye with tenuous growth in 4% NaCl while the growth was lacking in 6.5% NaCl and 45°C and produced acid and formed clot in litmus milk causing the lowering of pH from 6.5 to 4.5, while other isolates (Lc2, Lc3, Lc6, Lc7 and Lc9) did not produce ammonia from arginine due to the stability of the orange color of the medium, and lacked the ability to grow in 4%NaCl as well as inability to grow in 0.1% methylene blue, also, all isolates taken part in their inability to grow at 45°C and pH 9.5 but have grew at pH 9, facultative anaerobic since it had the ability to grow under aerobic and anaerobic conditions and can grow at (10-40)°C, these results are in agreement with taxonomic characteristics of the genus *Lactococcus* which were mentioned by [16].

In order to differentiate the nine isolates of *Lactococcus* species, carbohydrates fermentation test was performed. The isolates were different in their ability to ferment the carbohydrates source used. The isolates (Lc2, Lc3, Lc6 Lc7, Lc9) which fermented

(glucose, sucrose, lactose, mannose, mannitol, galactose, maltose and raffinose) but varied in their ability to ferment arabinose and xylose were identified as *Lactococcusraffinolactis*. While the isolates (Lc1, Lc4, Lc5, Lc8) vary in their ability to ferment xylose while unable to ferment arabinose and raffinose but have fermented the other used saccharides were identified as *L. lactis* [19].

According to the results above, the overall resultant was nine isolates of *Lactococcus*; five of them (Lc2, Lc3, Lc6 Lc7 and Lc9) were identified as *Lactococcus* sp. *Raffinolactis* and the others (Lc1, Lc4, Lc5, Lc8) were identified as *Lactococcus* sp. *lactis*. Our study focused on studying the effect of the crude extracts of the genus *Lactococcus* sp. *lactis* ignoring the species *raffinolactis.*

Table (2): Biochemical tests of the locally *Lactococcus* Isolates.

Isolate	Catalase Test	Gelatinas Test	NH_3 product. from Arginin	Growth in litmus Milk	Growth at			Growthin 1% methylene blue	Acid Production from									
					10 °C	40 °C	45 °C		Glucose	Sucrose	Lactose	Mans	Manl	Arab	Xyl	Gal	Mal	Raf
Lc1	-	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-
Lc2	-	-	-	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+
Lc3	-	-	-	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+
Lc4	-	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-
Lc5	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	-
Lc6	-	-	-	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+
Lc7	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+
Lc8	-	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-
Lc9	-	-	-	+	-	+	-	-	+	+	+	+	+	+	-	+	+	+

Glu: glucose. Suc: sucrose. Lac: lactose. Mans: mannose.Manl: mannitol.
Arab: arabinose. Xyl: xylose. Gal: galactose. Mal: maltose. Raf: raffinose.

The standard curve of bovine serum albumin was plotted and figure (1) explains this curve.

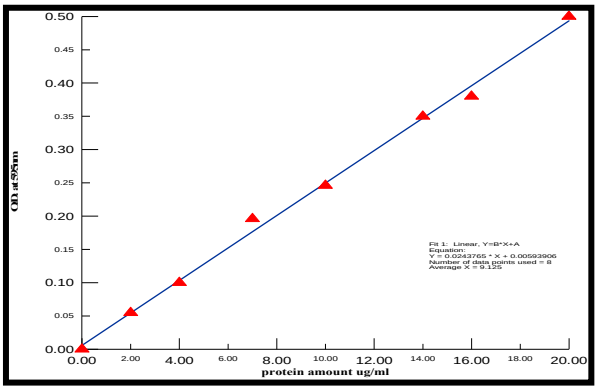

Figure (1): Standard curve of Bovine Serum Albumin.

Extraction and Purification of Extracted Proteins

The results were three extracts (one from each medium) in addition to CWP; these extracts have given termed according to the production medium and the saturation ratio with the ammonium sulphates as follow:

- M6-80: Proteins extracted from *L. Lactis* grown in MRS broth for 6 hr with 80% ammonium sulphate saturation ratio.
- B6-80: Proteins extracted from *L. lactis* grown in BHI broth for 6 hr with 80% ammonium sulphate saturation ratio.
- V6-80: Proteins extracted from *L. lactis* grown in VVM broth for 6 hr with 80% ammonium sulphate saturation ratio.

Table (3): The Concentrations of total proteins in supernatants of the locally isolate *L. lactis* protein production media.

TheCrude Extracts	O.D. (595 nm)	Protein concentration (µg/ml)
M6-80	0.401	16.226
B6-80	0.330	13.299
V6-80	0.372	15.034

The Concentrations of Extracted Proteins

When compared with standard curve of BSA, the concentrations of extracted proteins were variant; these variations may be due to the type of production media and saturation ratio with ammonium sulphate. The production was highest in MRS broth and this compatible with Boutrou [20] who observed that several of the proteins were present in abundance in the supernatants of mid-log-phase cultures of MRS broth medium when compared with other production media such as GM17 and chemically defined medium (CDM) containing glucose, this probably due to the inactivation of the proteins by the proteolytic / peptidolytic system of the producer cells by the ingredients of these media. Along with Lei *et al*., [21], the lesser production was in the BHI broth medium who observed that the production of proteins was lower in BHI broth supplemented with erythromycin 10 µg/ml or spectinomycin 100 µg/ml when compared with that of Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml), Table (4).

Table (4): The Concentrations of total proteins extracted from the locally isolate *L. lactis* after precipitation with ammonium sulphate.

The Crude Extracts	O.D.(595 nm)	Protein concentration (µg/ml)
M6-80	0.331	13.572
B6-80	0.251	10.053
V6-80	0.302	12.145

On the other hand, Partial purification of the proteins was carried out by precipitation with ammonium sulphate. In addition to purification, this step leads to considerable lose in the concentrations of extracted protein when compared between Table (3,4) [22].

The Effects of the Crude Extracts of *L. lactis* on AMN-3 Cell Line

The results showed that there was an obvious cytotoxic effect for these concentrations in the growth of AMN-3 cell line during the three periods of incubation that has started at certain concentrations and continued to the higher concentrations reaching the last concentration when compared with the control. Also, it was observed that there were variations in cytotoxic effect between one extract and another and among the concentrations of the same extract but at different periods of incubation, Table (5).

Table (5): The concentrations of the crude extracts of the locally isolate *L. lactis* at which the cytotoxic effect on AMN-3 cell line has started after incubation for different periods.

The Crude Extracts	The concentrations (µg/ml) at which the cytotoxic effect has started after:		
	24 h	48 h	72 h
M6-80	1.25	0.625	0.625
B6-80	2.5	1.25	0.625
V6-80	1.25	0.625	0.625

The Effect of the Crude Extract M6-80

As shown in Table (6), this crude extract has cytotoxic effect in the growth of AMN-3 cell line starting at the concentration 1.25 µg/ml with high significance difference (P≤0.001) accompanied with increased cytotoxic effect toward the higher concentrations when compared with the control (the same cell line treated with SFM only) during the incubation period 24 hr. The concentration 0.625 µg/ml has cytotoxic effect in the growth of this cell line at 48 hrs incubation period with high significant difference (P≤0.001) with increased cytotoxic effect toward the higher concentrations when compared with the control. While during the incubation period 72 hrs, the cytotoxic effect has started at the concentration 0.625 µg/ml with high significant difference (P≤0.001) with increased cytotoxic effect toward the higher concentrations when compared with the control.

Table (6): The effect of M6-80 on AMN-3 cell line

Group	Conc. (µg/ml)	O.D. Mean ± (SE x 10^{-3}) at different incubation periods		
		After 24 hrs	After 48 hrs	After 72 hrs
Treated	0.312	0.44100 ± 3.215 [a]	0.42533 ± 3.845 [a]	0.40433 ± 3.285 [a]
	0.625	0.43500 ± 2.673 [a]	* 0.38900 ± 7.638 [b]	* 0.37200 ± 8.082 [b]
	1.25	* 0.40700 ± 3.054 [b]	0.37433 ± 6.766 [b]	0.32400 ± 5.565 [c]
	2.5	0.39400 ± 0.011 [b]	0.32067 ± 8.873 [c]	0.29533 ± 4.255 [d]
	5	0.36100 ± 5.294 [c]	0.27700 ± 5.860 [d]	0.29100 ± 6.027 [d]
	10	0.27900 ± 5.132 [d]	0.25133 ± 7.309 [e]	0.21600 ± 4.041 [e]
Control I		0.44500 ± 1.997	0.43200 ± 2.644	0.41300 ± 3.510

*different letters= significant differences (P<0.05) between mean.

The Effect of the Crude Extract B6-80

Cytotoxic effect of this crude extract on the growth of AMN-3 cell line has obviously appeared after 24 hrs of incubation with high significant difference (P≤0.001) at the concentration 2.5 µg/ml with increased cytotoxic effect toward the higher concentrations when compared with the control. At 48 hrs incubation period, the cytotoxic effect has started with significance difference (P≤0.001) at the concentration 1.25 µg/ml with increasing toward the higher concentrations when compared with the control. While during the incubation period 72 hrs, the cytotoxic effect with significance difference (P≤0.001) has started at the concentration 0.625 µg/ml attended with increased cytotoxic effect toward the higher concentrations when compared with the control, Table (7).

Table (7): The effect of B6-80 on AMN-3 cell line

Group	Conc. (µg/ml)	O.D. Mean ± (SE x 10^{-3}) at different incubation periods		
		After 24 hrs	After 48 hrs	After 72 hrs
Treated	0.312	0.44567 ± 5.455 [a]	0.44200 ± 5.565 [a]	0.43400 ± 5.565 [a]
	0.625	0.43000 ± 6.246 [a]	0.41800 ± 5.565 [a]	* 0.40200 ± 7.095 [b]
	1.25	0.42200 ± 8.019 [a]	* 0.37400 ± 7.095 [b]	0.38000 ± 6.558 [c]
	2.5	* 0.39700 ± 2.517 [b]	0.38600 ± 4.728 [b]	0.35000 ± 7.026 [d]
	5	0.37600 ± 5.132 [c]	0.36400 ± 5.565 [c]	0.28067 ± 6.010 [e]
	10	0.32300 ± 7.026 [d]	0.28400 ± 5.132 [d]	0.24567 ± 5.455 [f]
Control I		0.44700 ± 4.162	0.44167 ± 3.931	0.43567 ± 5.445

*different letters= significant differences (P<0.05) between mean.

The effect of the crude extract V6-80

Cytotoxic effect of this crude extract has started with high significant difference (P≤0.001) at the concentration 1.25 µg/ml toward the higher concentrations when compared with the control during the incubation period 24 hrs. At incubation period 48

hrs, cytotoxic effect with high significant difference (P≤0.001) was at the concentration 0.625 µg/ml toward the higher concentrations when compared with the control. While during the incubation period 72 hrs, the cytotoxic effect with high significant difference (P≤0.001) has started at the concentration 0.625 µg/ml with increased cytotoxic effect toward the higher concentrations when compared with the control, Table (8).

Table (8): The effect of V6-80 on AMN-3 cell line

Group	Conc. (µg/ml)	O.D. Mean ± (SE x 10^{-3}) at different incubation periods		
		After 24 hrs	After 48 hrs	After 72 hrs
Treated	0.312	0.46800 ± 2.084 [a]	0.45667 ± 3.279 [a]	0.42200 ± 5.132 [a]
	0.625	0.45833 ± 2.904 [a]	* 0.41400 ± 4.041 [b]	* 0.38400 ± 5.686 [b]
	1.25	* 0.42600 ± 4.584 [b]	0.41500 ± 3.510 [b]	0.32000 ± 6.656 [c]
	2.5	0.42267 ± 5.363 [c]	0.36500 ± 5.565 [b]	0.27200 ± 7.551 [c]
	5	0.39000 ± 3.510 [d]	0.32000 ± 0.010 [b]	0.25400 ± 4.509 [d]
	10	0.35200 ± 2.084 [e]	0.30900 ± 6.108 [c]	0.20000 ± 9.607 [e]
Control I		0.47000 ± 4.584	0.46200 ± 4.162	0.45900 ± 5.034

***different letters= significant differences (P<0.05) between mean.**

Our findings consistent to those gained by [23] who showed that *Streptococcus pyogenes* (group A *Streptococcus*) cell extracts (CE) at concentrationsabove 0.5 µg/ml among the used concentrations (0, 0.008, 0.04, 0.2, 0.5, 1.0, and 5.0µg/ml) consistently caused potent inhibition of T-cellproliferation *in vitro* during three days incubation period. Streptococcal acid glycoprotein (SAGP); this protein possesses between 31.5 and 39.0% amino acid identity with arginine deiminase (AD) with native molecular mass has been reported as 140 to 150 kDa and also 220 kDa [24]. AD is one of three enzymes that comprise the AD system. AD catalyzes the conversion of L-arginine to citrulline, with the concomitant production of ammonia. The AD system is widely distributed among prokaryotes, including *Enterococcus faecalis*, *L. lactis*, *Enterococcus faecium*, and *Clostridium perfringens*, and in *Mycoplasma* the catabolism of L-arginine by this enzyme complex acts as a major nonglycolytic metabolic energy source. Purified group A streptococcal AD have all been shown to be dimers composed of two identical subunits with molecular masses in the range of 46 to 54 kDa [18]. AD has been well documented as having antiproliferative activity against a range of tumor cell lines, including murine fibrosarcoma Meth A cells, human HL60 cells, murine embryonic cells (BALB/3T3), HeLa cells, and murine leukemic L1210 cells, SAGP has been used clinically as an antitumor agent, but it appears to mediate its tumouricidal effect by modulating the host immune response through pathways not involving the activity of SAGP. Lyophilized preparation of SAGP (OK-432) is known to activate natural killer cells, T cells, and macrophages *in vitro*, and animals treated with OK-432 intraperitoneally develop antitumor cytotoxic macrophages, also, SAGP is reported to have a direct cytotoxic effect on tumor cell lines [25]. *In vitro*, L-arginine is essential for the optimal growth and proliferation of cells, but lack of extracellular L-arginine in the growth medium is thought not to lead to cell death. The action of AD will lead to a depletion of L-arginine in growth media, and may be in the absence of L-arginine cells are simply unable to synthesize new proteins, thus inhibiting growth and proliferation [23]. Since *L. lactis* is a genus of group A streptococci so their inhibitory effect in the growth of tumor cell lines may be attributed to this active compound (SAGP).

Cancer cells possesses metabolic characteristics unavailable in normal cells; including opportunism, fastidious nutrition requirements and the ability for invasion and distribution, this require the presence of specific genes or proteins differ from those of normal cells so, the cancer cells perhaps be a target for the active compounds of the secondary metabolites e.g., the formation of free radicals during lipid peroxidation which is considered an important step to provide energy during the transformation of the normal cells to cancer cells [26]. So, the active components of the secondary metabolites of the studied bacteria may play an important role in their cytotoxic effect in cancer cells by retarding the activity of lipoxygenase via inhibition 5-lipoxygenase mRNA which is overexpressed in cancer cells, that by role inhibits topoisomerase I responsible for DNA replication and then cell proliferation which lead the cell to reach PCD [27].

The effect of the crude extract of *L. lactis* on normal rabbit embryo fibroblast (REF)

Statistical analysis has showed that there is no significant difference ($P \leq 0.039$) for each concentration of these crude extracts when compared with both control I and control II indicating that these crude extracts have no lucid effect on the growth of REF cell line after incubation period for 72 hrs, Table (9).

Table (9): A comparison between the effect of the crude extracts of *L. lactis* on AMN-3 cell line and REF for 72 hrs

Group	Conc. (µg/ml)	O.D. Mean ± (SE x 10^{-3}) at 72 hrs M6-80	B6-80	V6-80
Treated	0.312	0.40433 ± 3.285 [a]	0.43400 ± 5.565 [a]	0.42200 ± 5.132 [a]
	0.625	* 0.37200 ± 8.082 [b]	* 0.40200 ± 7.095 [b]	* 0.38400 ± 5.686 [b]
	1.25	0.32400 ± 5.565 [c]	0.38000 ± 6.558 [c]	0.32000 ± 6.656 [c]
	2.5	0.29533 ± 4.255 [d]	0.35000 ± 7.026 [d]	0.27200 ± 7.551 [c]
	5	0.29100 ± 6.027 [d]	0.28067 ± 6.010 [e]	0.25400 ± 4.509 [d]
	10	0.21600 ± 4.041 [e]	0.24567 ± 5.455 [f]	0.20000 ± 9.607 [e]
REF		0.43087 ± 4.361 [a]	0.42398 ± 6.393 [a]	0.45879 ± 5.483 [a]

***different letters= significant differences (P<0.05) between mean.**

There was no effect or slight effect without significance on the growth of REF cell line, this selectivity may be attributed to the metabolic behavior possessed by cancer cells rather than in normal cells such as metabolic nature to form new blood vessels, the shape and nature of the receptors presents on the surface of cancer cell and the ability to bind with different compounds [28]. In addition, in cancer cells DNA strands are relaxant and the whole molecule is unstable due to the distance between the hydrogen bonds (H-bonds) that bind the two strands together this facilitates the binding of intra- and extra-compounds with DNA strands while in normal cells DNA molecule is cohesive and H-bonds are close to each other this prevent the binding of other foreign compounds [29].

Conclusions

The crude extracts M6-80, V6-80 and B6-80 of *L. lactis* possess conspicuous cytotoxic effect in growth inhibition of AMN-3 tumour cell line *in vitro* depending on both concentration and time. On the other hand, these crude extracts have no cytotoxic effect with observed significance differences in the growth of normal rabbit embryo fibroblast (REF) cell line.

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