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Potential Cytotoxic Effect of Crude Extract Produced by *Lactobacillus spp* on Caco-2 & L20B Cell Lines *in vitro*

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Abstract:

This study was designed to determine the cytotoxic effect of crude products which was produced by isolates of Lactobacillus spp against three cell lines using 3-(dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT assay). About 72 samples were collected from local dairy products and infant feces with age of (1- 40 days) in Baghdad, the collection of those samples last long (September 2016 to January 2017). All isolates were identified as Lactobacillus spp according to their morphological, cultural, biochemical features and PCR technique by 16SrRNA gene. The cytotoxicity of crude extract was evaluated on cancer intestinal cells of mice L20B, human colon adenocarcinoma Caco-2 and human hepatic WRL-68 cell lines. The results showed that crude extract had cytotoxic effect on L20B with IC50= 17 □g/ml and on Caco-2 with IC50=221.9 □g/ml in concentration 400 □g/ml for both. While the crude extract of WRL-68 cells did not show when treated with the same concentration when used WRL-68 as normal cells for comparison. Conclusion: In last years, the high prevalence rate of many types of cancer in Baghdad, especially of intestinal cancer in human, so had used materials was produced by Probiotic bacteria to study their effects on the cancer cells in vitro.

Key words: *MTT assay, Caco-2 cell line, L20B cell line, Crude extracts, Lactobacillus spp.*

Introduction:

The Arab countries including Iraq had a great number of cancer cases, with 14.9 million cases and 8.2 million deaths as reported in 2013[1]. An increased incidence of cancer increases with age. Many reasons may lead to increase the rate of colorectal cancer in human such as excessive alcohol consumption which lead to an increase the rate by 60%, while smoking and insufficient fiber intake increase the colorectal cancer incidence rate by 20%, other research had found that, the excessive intake of high-fat diets can also lead to colorectal cancer [2]. Through the last decades, the studies had shown that cancer cells have a unique metabolism compared with normal cells [3]. Metabolic changes take place in cancer cells are fundamental for the transformation of normal cells into cancer cells. Also, are responsible for the resistance to various types of chemotherapeutic drugs [4]. Thus, the resistance of chemotherapy represents the main problem in the treatment of several tumor types [5].Anticancer or chemotherapy drugs are powerful chemicals that kill cancer cells by arresting their growth at one or more than checkpoints in their cell cycle. Despite of chemotherapy agents can rapidly affect dividing cells, the normal cells are affected too; as a result, the side effects are observed with several types of chemotherapies [6].

The Probiotics are defined as live microorganisms which prove and support the immune of gut to their hosts when they were ingested in sufficient quantities [7]. The selection of probiotic include safety (must be a usually recognized as a safe strain), ability to adhere to the intestinal tract, acid and bile salt tolerance and exhibit health benefits to the host [8,9].

There are many effects of lactobacilli, when reduce the activity of tumor-promoting enzymes, produce metabolites that benefit the host, increase immunity of the host and resist pathogens. In in vitro studies, most models of human intestinal cells have been used to study the of specific intestinal cells function in humans. As an example Caco-2 was used cell line of human intestinal [10].

The mechanisms inhibitory of Lactobacillus against cancer cells (Caco-2 cell lines) reducing tumor-promoting enzymatic activity, increasing short-chain fatty acids, binding to muta-

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gens, enhancing immunity and lowering pH [11,12].Our study aspires to investigate the characteristics of probiotic and their ability to inhibit the growth of cancer cell lines Caco-2 and L20B.

Materials and Methods:

1. Bacterial Strains Matainaince:

Total of 8 strains of Lactobacillus, including Lb.11, Lb.13, Lb.16, Lb.20, Lb.21, Lb.48, Lb.59 and Lb.67 were obtained from different dairy products sources and identify as Lactobacillus spp according many tests involves, microscopic test, biochemical test[13] and genetic detection[14]. To prepare Lactobacillus strains for use in this study, the strains inoculated in Man Rogosa Sharpe medium (MRS broth), and activated tow times, after that incubation it at 370C for 20 hours under anaerobic conditions in an aerobic jar before use. The isolates kept in refrigerator at 4oC.

2. Preparation of Lactobacillus cells extracts:

The MRS broth was inoculated with 105-6 CFU.ml-1 strains culture [15]. After incubation, the cultures were centrifugation (5000 g/15 min/2°C). The pH of the cell-free supernatant was adjusted to 7 with 0.1M NaOH. The cell-free super¬natant was filter-sterilized with a micro-filter (0.22 µm; Millipore) and then the supernatant stored at 4°C until used in the assay [16].

3. Cancer Cell Lines Culture:

Two cancer cell lines (Caco-2 and L20B cells) and normal cell line WRL-68 were grown in Roswell park memorial institute (RPMI) 1640 medium. The cell line was prepared according the methods in the Research of Biotechnology center/ Al-Nahrain University. The media were supplemented with 25 mM glucose, 10% inactivated fetal bovine serum, and 1% penicillin/strepto¬mycin, and maintained at 37°C in a humidified incubator with 5% CO2. The cells with 20-30 passages were used for further investigations [17].

4. Cytotoxic activity (MTT Assay) and IC50 values of potential Bacterial cell extracts:

Anti-cancer activity of 8 potential Lactobacillus cell extracts was carried out using (MTT assay) 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-di-phenyltetrazolium bromide. The assay measured the formation of blue formazan product as a result of the reduction of MTT by mitochondrial dehydrogenase that indicates the normal function of mitochondria and cell viability [18, 19]. For the determination of IC50 values, strains cell extracts were dried, weighed and serially diluted in RPMI 1640 media, to achieve the following concentrations (400, 200,100, 50, 25, 12.5, 6.25 □g/ml). Concentrated crude cell extract was added (100 µg/well) to the wells of each cancer cell line beside normal cell lines with triplicate. Cells were incubated at 5% CO2 concentra¬tion for 24h at 37°C. Then 20 µl of PBS containing 5□g/ml MTT was added in each well. Then, contact with the MTT solution, after 4 h, at 37°C, then formed the formazan blue crystals and then they were dissolved in 100 µl DMSO. Reduced MTT was measured at 570nm using a microplate reader (test wavelength).Untreated cells were used as a negative control. The cytotoxicity of the tested supernatants was determined by comparing the absorption of the treated cells against the absorption of the control (untreated cells):

{1– [(absorbance of sample)/ (absorbance of control)]} × 100

Commonly, MTT assay was used to determine IC50 values that were the concentrations which show 50% inhibition of any tested cell line [20].

5. Isolation of DNA and PCR Primers and conditions:

DNA was extracted from Lactobacillus strains, DNA mini kit Geneaid"presto" (Korea) according to the manufacturer's instructions, which efficiently releases DNA from gram-positive organisms cultured under anaerobic conditions. According to Sambrook and Russel [21] agarose gel of 1.5 % concentrations was utilized to confirm the size of genomic DNA bands and to confirm the size of the PCR products.

The agarose gel consists of 1.8 g dissolved in 120 ml of 1X TBE buffer using a microwave. After the agarose solution cools down to 55-60 Cº, a 1 µl of 0.5 µg/ml final concentration ethidium bromide (EtBr) was added. Then, the solution was poured into the gel tank with the combs in place and let to cool for 30 min. The combs were removed carefully and tank was placed in the electrophoresis system containing running buffer consisting of 1X TBE, the buffer is poured until it covers the gel for about 1-2 mm. Ten microliter of each PCR product along with the negative control and DNA ladder (100 bp) were loaded into the wells, the system cover was then placed and the system was turned on. Electrophoresis was performed for 1hr with a 70 volt/35 mAmp current. The DNA bands were visualized with a UV transilluminator was followed and photographed by using digital camera.

6. Statistical Analysis:

In present study, used Graphed 6 Prism Program for calculation of our data to analysis. One- way ANOVA followed by Dennett's multiple comparisons test. Data represents the mean ±SD of three independently repeated experiments [22].

Results and discussion:

1. Identification of Lactobacillus spp by 16SrRNA gene:

Identification of the isolates as Lactobacillus spp, was done by Polymerase Chain Reactions (PCR) using specific primers Lacto F and Lacto R designed by McOrist et al.[14](table 1).

Table (1): PCR primers [23].

Primer	Sequence (5_-3_)	Specificity	Location[a] (5-_3-)	Region of 16S rRNAb	Annealing temp (C⁰)	Amplicon size[c] (pb)
LactoF	TGGAAACAGRTGCTAATACCG	All lactobacilli	157-176	V2.1-V2.2	62	231-233
LactoR	GTCCATTGTGGAAGATTCCC	All lactobacilli	379-360	V2.2-V3		

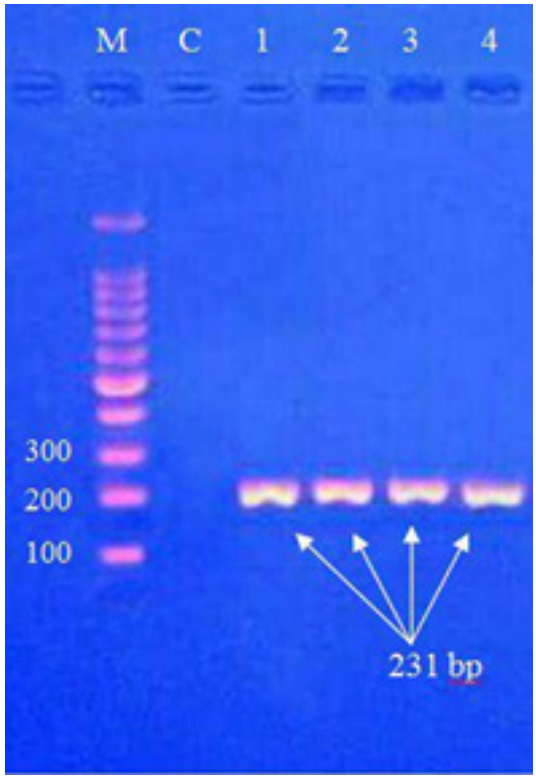

Figure (1): Gel electrophoresis for amplification genes encoded to 16SrRNA in Lactobacillus isolates. Electrophoresis was performed on 1% agarose gel and run with a 5v/cm current for 2 hrs. With (1500bp) ladder, Line C: P.aeruginosa, 1, 2, 3 and 4 Lactobacillus isolates.

The PCR primers that amplified DNA only from bacteria belonging to the Lactobacillus group since it can anneal to 16SrRNA of this genus figure (1).In this study, showed that a Lacto F and Lacto R primers -based PCR could be a useful tool for identification of the members of the Lactobacillus genus.

2. Cytotoxicity effect of Lactobacillus spp:

Current study aimed at screening the cytotoxic activity for cell-free culture supernatant (CFCS) of Lactobacillus spp against L20B, CaCo-2 and WRL-68 cell line, by MTT assay in vitro.

Crude extract from Lactobacilli bacteria, that isolated from various sources tested for anticancer activity by measuring viability cells using MTT assay. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt converted to an insoluble purple formazan by the cleavage of the tetrazolium ring through the succinate dehydrogenase within the mitochondria. Since the cell membranes are impermeable to the formazan product, the product accumulates, in healthy cells [24].The results showed variable antitumor activity on cancer cell line L20B, which isolated from mice intestine (Albino mice).The ratio for CFCS of Lb. 21, that isolated from local cheese which made by traditional method, showed higher inhibition effects, whereas the viability of cells were (32.7%, 59.2%, 91.7% and 95%) in concentrations (400, 200, 100 and 50) □g/ml, respectively; and IC50= 17 □g/ml figure (2).

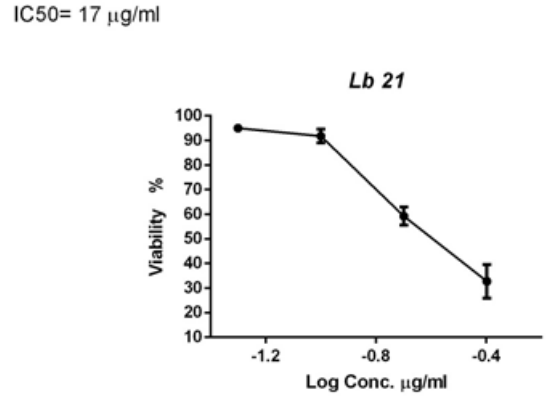

Figure (2): IC50 value of Lb. 21- treated L20B cells after 24 hours of incubation at 37oC.

Also, the study explained that Lb. 21 isolated have higher anticancer effect tested with Caco-2 cell line. The extract of this strain showed (52.85%, 65.58%, 78.16%, 79.75%, 83.79%, 86.29%) at increase the concentrations (400, 200, 100, 50, 25, 12.5, 6.25 □g/ml) respectively with IC50=221.9□g/ml figure(3), while there was no clear effect for WRL-68 cell line even in high concentration as in figure (3).

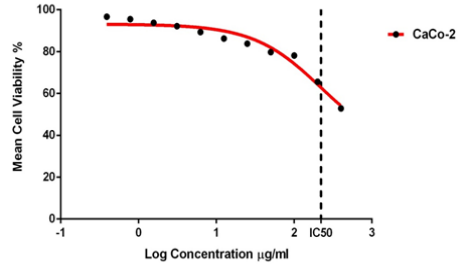

Figure (3): Cytotoxic effect of Lb.21 on CaCo-2 cell line with IC50=221.9 □g/ml.

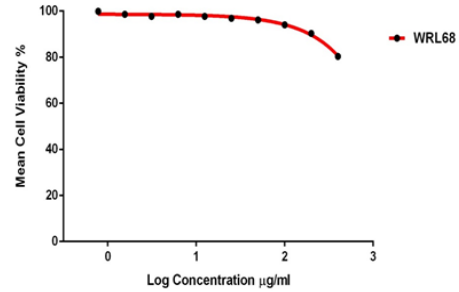

Figure (4): Viability cells of Lb.21 on WRL-68 cell line.

In table (2), results showed that inhibition percentage caused by CFCS in different concentrations for both Caco-2 and WRL-68 cell line in vitro. Results revealed that CFCS inhibited between (47.15 –13.71%) from viable cells in Caco-2 cell line in concentration ranged between (400 –12.5 □g/ml), with IC50= 221.9 □g/ml.

While in normal cell line WRL-68 the inhibition percentage was (19.64, 9.68, 5.94, 3.8, 3.1 and 2.3) in concentrations (400, 200, 100, 50, 25 and 12.5) respectively, as in figure (4).

Table (2): cell viability of Lb.21 supernatants on (CaCo-2 and WRL-68) cell lines.

CaCo-2	WRL-68		
Concentration (mg/ml)	**400**	52.85 ± 7.580	80.36 ± 3.155
	200	65.58 ± 9.869	90.32 ± 4.156
	100	78.16 ± 8.541	94.06 ± 2.376
	50	79.75 ± 8.735	96.20 ± 3.466
	25	83.79 ± 7.450	96.90 ± 1.456
	12.5	86.29 ± 6.175	97.70 ± 0.3342

In this study, we evaluated anti proliferative effects of the cell-free culture supernatants (CFCS) of several Lactobacillus strains on the cancer intestine albino mice cells L20b, the human adenocarcinoma cell line Caco-2 and normal hepatitis cell line WRL-68.

CFCS of Lactobacilli were found to inhibit the growth of colon cancer cells in a dose-dependent manner as detected by the MTT assay.

We adopted on the best a dose to inhibit growth of the cancer cells for L20B and Caco-2 cells. In the same time this not effect on normal cells, which was WRL-68 as an example for natural cells. Other studies they adopted the best times which inhibited cancer cells [25].

The reasons of inhibit growth of the cancer cells may back to several substances, which produced by Lactobacillus in best medium and an optimum conditions in vitro.

CFCS of Lactobacillus was containing various metabolites. Bacteriocin is an important product that, produced by different species of Lactobacillus [25]. The action mode of bacteriocin was binding of proteins to lipid II, and the peptidoglycan subunits not transport to the wall of cell, and this leads to synthesis of cell wall with incorrect way, therefor results the death of cell [26].

Frequently, in vitro the most used tests are those that are conducted to measure the metabolic activity of viable cells by colorimetric changes based on tetrazolium salt reduction. For example, peptidoglycans isolated from L. casei were found to have anticancer activities against various human cancer cell lines that involve Caco-2 [27]. Additionally, in a study provided by Sevda et al. the antiproliferative effects of the cell-free filtrate and the cell-free lyophilized filtrate of LAB cultures were reported against Caco-2 cell lines [28].

At the examination level, Caco-2 (colon adenocarcinoma) cell line is one of the most used in vitro models for the study of intestinal absorption of compounds. Despite of the vivo processes are mostly complex may cast some doubts on the full representativeness of the model; it has become a popular surrogate for human intestinal epithelium [29].For this cause, we used Caco-2 cells in our study.

Lactobacilli and bifidobacteria are mostly used as probiotic bacteria, for this reason has been accepted for the increasing research attention on the prevention of cancer [30].

The results of this study shown that lactic acid bacteria produced metabolic substances that affect in that cancer cells L20B and Caco-2 (in vitro) at a concentration of 400 □g/ml and this concentration does not affect the growth of normal cells WRL-68, which confirms the safety of the use of this type of Probiotics bacteria in dairy products. So it is recommended consumption of dairy products which rich in Probiotics bacteria daily, especially those have a genetic history of bowel cancer.

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