Cytotoxic Effect of *Lactobacillus gasseri* Concentrated Filtrate on some Tumor Cell Lines *in vitro*

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Abstract

This study was designed to investigate the cytotoxic effect of *Lactobacillus gasseri* concentrated filtrate on tumor cell lines. *Lb. gasseri* was grown in de Mann, Rogosa and Sharp MRS broth medium and incubated anaerobically at 37ºC for 24 hrs. The culture was centrifuged and the supernatant was taken and sterilized by filtration. One hundred ml of filtrate was concentrated by oven at 40-45 ºC to three- fold (12.5 ml) and different concentrations (125, 250, 500, and 1000) µg/ ml were applied on RD, Hep-2, and AMN-3 tumor cell lines and normal cell line REF and incubated at 37ºC for 24 hrs. Results showed that the growth of RD and HepG2 were significantly inhibited ($p \leq 0.05$) at125 µg/ ml of filtrate and the percentage of growth inhibition was (91.9 and 86.4)% respectively. The effect was dose-dependent and accordingly 1000µg/ ml recorded the highest percentage of growth inhibition, which was 27.8% on AMN-3 cells and 32.1% on REF cell line.

Keywords: *Lactobacillus gasseri*, Filtrate, Cytotoxic effect.

Introduction

Lactic acid bacteria (LAB) such as *Lactobacillus* are important micro-organisms in a healthy human microbiotic environment [1]. LAB is beneficial micro-organisms, which have been associated with several probiotic effects in both humans and animals [2]. Numerous reports have indicated that both LAB and fermented milk exert anticancer effects [3,4]. Data from epidemiological and experimental studies have also indicated that the ingestion of certain LAB strains, or of fermented dairy products, might alleviate the risk of certain type of cancers, and inhibit the growth of tumors [5]. Many strains, including *Lb. rhamnosus GG, Lb. acidophilus*, *Lb. casei*, *Bifidus longum*, *Bifidus infantis*, *Bifidus adolescentis*, and *Bifidus breve*, suppress experimental colon tumor incidence [6, 7].The precise mechanism by which LAB exerts anticancer effects has been previously reported to induce apoptosis [8]. Apoptosis (programmed cell death) is frequently impaired in many human tumors, and is also an important phenomenon in chemotherapy-induced tumor cell death. Therefore, the modulation of apoptosis via the targeting of pro-apoptotic and anti-apoptotic proteins may prove to be an effective technique in the treatment of cancer [9].

Materials and Methods

Bacterial isolate

Lb. gasseri was supplied by Immunology Lab., Department of Biotechnology, College of Science, Al-Nahrain University. This isolate was previously isolated from red peach fruit (*Prunus persica*).

Cell culture

Three types of cancer cell lines, liver carcinoma cell line (HepG2), Rhabdomyosarcoma cell line (RD), Mice mammary gland carcinoma cell line (AMN), and normal cell line Rat fibroblast cell line (REF) were kindly obtained from Iraqi Center for Cancer Research and Medical Genetics (ICCRMG) and used in this study. Cells were grown at 37 ºC in humidified atmosphere containing 5% CO_2 in RPMI- 1640 medium supplemented with 10% fetal calf serum(FCS), Glutamine (2Mm), penicillin(100IU/ml), and streptomycin (100µg/ml) [10].

Preparation of concentrated filtrate of *Lb. gasseri*

Tube containing de Mann, Rogosa and Sharp MRS broth medium was inoculated with 1% of fresh culture *Lb. gasseri*, then incubated anaerobically at 37°C for 24 hrs. After incubation, culture was centrifuged at 6000 rpm for 15 min., supernatant was taken and sterilized by Ultrafiltration [11]. One hundred ml of filtrate was concentrated by oven at 40-45 °C to one fold (50 ml), two -fold (25 ml) and three- fold (12.5 ml). A portion of 100mg from each of *Lactobacillus spp.* concentrated filtrate was dissolved in 10 ml of serum free medium, then sterilized by Millipore 0.22µm filter. From these stock solutions, two fold dilutions were made starting from 1000µg/ml to 125µg/ml.

Cytotoxic assay

Cytotoxic assay was carried out according to Freshney [10]. Cell suspension was prepared for each type of cell lines and 1×10^5exponentially growing cells seeded in 96 well tissue culture plates as(200 µL) in each well and incubated at 37 ºC for 24hrs. After incubation, the wells were examined for the formation of cell monolayers and 200 µl/ well from each concentration (125, 250, 500, or 1000) µg/ml of three fold concentrated filtrate were added to the wells as three replicates for each concentration. Three replicates were made for control which contained only the cells growing on growth medium. After 24 hrs, 50 µl/ well of neutral red dye was added and incubated again for 2 hrs. After incubation, the contents of the plate were removed by washing the cells with PBS to remove the excess dye followed by the addition of 20 µl/well of extraction dye solution that draw out the dye from the viable cells that had stained. Results were read using ELISA reader at a wave length 492 nm. The percentage of growth inhibition (PGI) was calculated according to the following equation [12].

$$\text{Growth inhibition (\%)} = \left(\frac{\text{Control Absorbance - Treated Absorbance}}{\text{Control Absorbance}}\right)\times 100$$

Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA), Duncan test, using SPSS computer program version (7.5) was performed Differences in results were considered significant at probability value equals or less than 0.05 [13].

Results

Results revealed that four concentrations of *Lb gasseri* three fold concentrated filtrate were effective in reducing the growth of RD cell line. The lowest concentration recorded highest percentage of growth inhibition reached 91.9% compared with control, while, the highest concentration showed the lowest PGI recording 28.9% (Table (1)).

Table (1)
Growth inhibition effect of Lb gasseri concentrated filtrate on RD cell line after 24 hrs of incubation period.

Concentration (µg/ml)	***O.D. mean ± S.E.****	***growth inhibition (%)***
Control	0.619± 0.02 a	----
125	0.050±0.02 b	91.9
250	0.400±0.032 c	35.3
500	0.435±0.02 d	29.7
1000	0.440±0.03 e	28.9

different letters= significant differences (P≤0.05) between means and control treatment only.

According to the results indicated in Table (2), the first concentration of *Lb gasseri*, three fold concentrated filtrate displayed a significant cytotoxic effect on HepG2 cell line since PGI for this concentration was 86.4% compared with control. Other concentrations (250, 500, and 1000) µg/ml were not significant PGI (17.1%, 4.0 %, and 4.2%) respectively.

Table (2)
Growth inhibition effect of Lb gasseri concentrated filtrate on HepG2 cell line after 24 hrs of incubation period.

Concentration (µg/ml)	*O.D. mean ± S.E.**	*growth inhibition (%)*
Control	0.450±0.02 a	----
125	0.061±0.02 b	86.4
250	0.373±0.02 a	17.1
500	0.432± 0.02a	4
1000	0.431±0.02 a	4.2

different letters= significant differences (P≤0.05) between means and control treatment only.

As shown in Table (3), results indicated that only 250, 500 and 1000µg/ml of *Lb gasseri* three fold concentrated filtrate had a significant cytotoxic effect on AMN cell line since PGI for these concentrations were 23.9%, 24.0%, and 27.8% compared with control. The first concentration had no significant cytotoxic effect and PGI for this concentration was 11.4%.

Table (3)
Growth inhibition effect of Lb gasseri concentrated filtrate on AMN cell line after 24 hrs incubation period.

Concentration (µg/ml)	*O.D. mean ± S.E.**	*growth inhibition (%)*
Control	0.610±0.02 a	----
125	0.540±0.02 a	11.4
250	0.461±0.03 b	24.4
500	0.463±0.02 c	24
1000	0.440±0.03 d	27.8

different letters= significant differences (P≤0.05) between mean and control treatment only.

Table (4) showed that the three fold concentrated filtrate of *Lb. gasseri* had significant cytotoxic effect on growth of REF at500 and 1000 µg/ml with PGI of (35% and 32.1%) respectively when compared with the control. The first and second concentrations exerted significant cytotoxic effect on normal cell line and PGI were 24.2% and 24.6% respectively.

Table (4)
Growth inhibition effect of Lb gasseri concentrated filterate on REF cell line after 24 hrs incubation period.

Concentration (µg/ml)	*O.D. mean ± S.E.**	*growth inhibition (%)*
Control	0.280±0.02 a	----
125	0.212±0.03 b	24.2
250	0.211±0.02 c	24.6
500	0.182±0.02 d	35
1000	0.190±0.03 e	32.1

different letters= significant differences (P≤0.05) between mean and control treatment only.

Discussion

Results revealed that three fold concentrated filtrate of *Lb. gasseri* showed cytotoxic effect on RD, Hep-G2 and AMN-3. This might be due to the secondary metabolites produced by LAB such as hydrogen peroxide (H_2O_2) which is capable of inducing apoptosis [14]. A similar result was obtained by Huang*et al.,* [15] who reported that direct exposure of human hepatoma cell line (SMMC- 7221) to hydrogen peroxide induces apoptosis characterized by morphological evidence and fragmentation of DNA assayed by terminal deoxynucleotidyl transferase assay and confirmed that H_2O_2 can also activate the differential expression of some specific gene such as p53. The p53 tumor suppressor gene is an essential mediator of the mammalian cells and it is required for apoptosis. Subsequently, p53 activates the transcription of several genes whose products are involved in DNA repair or apoptosis. Another study showed that glycoproteins detected in the supernatants of *Lactobacillus* cultures also have anti-tumor effects [16].

Moreover, this effect could be attributed to polysaccharides produced by LAB [17] as reported by Hyun *et.al.* [18] who showed that the polysaccharide fraction (BB-pol) extracted from *B. bifidum* BGN4 had a novel composition, comprising from chiroinositol, rhamnose, glucose, galactose, and ribose and inhibited the growth of HT-29 and HCT-116

cells but did not inhibit the growth of Caco-2 cells.

Kim *etal*. [19] showed that expression of Bcl-2 interacting protein and cell division cycle protein were significantly regulated by the polysaccharides isolated from *Lb. acidophilus* 606 in proteomic analysis.

Conclusion:

It is concluded from this study that three fold concentrated filterate of *Lb. gasseri* showed a cytotoxic effect on the three types of tumor cell lines and normal cell line under investigations.

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الخلاصة

اجريت الدراسة لمعرفة التأثير السمي لراشح بكتريا Lactobacillus gasseri على الخطوط السرطانية في الزجاج. نميت بكتريا Lb. gasseri في وسط MRS السائل لفترة ٢٤ ساعة على درجة ٣٧°م تحت ظروف لاهوائية، بعدها فصل الراشح وركز باستخدام الفرن الكهربائي على درجة ٤٠-45°م. حضرت اربعة تراكيز من الراشح المركز (١٢٥ و ٢٥٠ و ٥٠٠ و ١٠٠٠ مايكروكرام/ مل) ودرست الفعالية السمية ضد الخطوط السرطانية (Hep-RD, G2,and AMN) وخط من الخلايا الطبيعية REF لفترة حضانة

٢٤ ساعة على درجة ٣٧°م. اظهرت النتائج امتلاك التراكيز الواطئة 125 µg/ml من الراشح المركز فعالية سمية ضد Hep-G2 و RD حيث كانت النسبة المئوية للتثبيط 86.4% و 91.9% على التوالي، بينما اعطت التراكيز العالية 1000µg/ml من الراشح المركز فعالية سمية ضد AMN حيث كانت النسبة المئوية للتثبيط 27.8%. اظهرت النتائج ان النسبة المئوية للتثبيط ضد الخط الطبيعي REF عند التركيز 1000µg/ml كانت 32.1%.