Int.J.Curr.Microbiol.App.Sci (2020) *9*(5): 891-906

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 5 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.905.098

Cytotoxicity Effects of *Lactobacillus acidophilus* on Hep G2 Cell Line and its Effects on Gene Regulation with Bax and Bcl2

N. G. Ramesh Babu[1], K. A. Simrah Fathima[2]*, V. Nandhini[3] and V. Nandhini[4]

Department of Biotechnology, Adhiyamaan College of Engineering, Hosur, Tamilnadu, India

**Corresponding author*

Keywords

Probiotic; *Lactobacillus*; Antiproliferation; Hep G2; Bax and Bcl2 gene

Article Info

Accepted: 05 April 2020
Available Online: 10 May 2020

A B S T R A C T

The microorganisms most commonly used as probiotics are lactic acid bacteria, especially those of the genus *Lactobacillus*. In the present study, *Lactobacillus acidophilus* were isolated from two different pooled samples homemade curd and commercial curd, were characterized for their antiproliferative activity. The antiproliferative effects of the strains were investigated using the MTT assay with liver cancer (Hep G2) cell line. The results showed that the *Lactobacillus* strains had good antiproliferative effects in liver cancer cell line. Further the viability of cells was observed with the help of fluorescent microscopy by dual staining technique. *Lactobacillus acidophilus* can be considered as potential probiotic bacteria for humans because of their antiproliferation effect in cancer cells. In this study, effect of the samples on expression of Bax and Bcl2 gene was analysed by RT -PCR. β-Actin was chosen as an internal control to normalize the gene expression. The study indicate that the MTT assay for the strains of *Lactobacilus acidophilus* isolated from the sample has cytotoxicity effect. Based on the IC_{50} value, homemade curd showed better percent of inhibition than commercial curd and Bcl2 gene showed fold up regulation when compared to Bax gene.

Introduction

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. The most commonly used probiotics in the food industry are Lactic acid bacteria, including the genus *Lactobacillus.* Probiotics have become highly recognized as supplements for human consumption because of their beneficial effects on health and well-being. For example, interference with pathogens and the prevention of infections, immune system stimulation and immunomodulation, anti-carcinogenic and antioxidant activities, reduction of gastrointestinal disorders such as diarrhoea, constipation and the irritable bowel syndrome, alleviation of allergic and lactose intolerance symptoms, reduction in serum cholesterol, blood pressure and risk of gestational diabetes (Dicks LMT and Botes M, 2010).

Among these effects, the antiproliferative or

cytotoxic effect of probiotic strains against cancer cells is a very important and relatively recent aspect. This probiotic property is important because cancer is considered as the major course of worldwide morbidity (Parisa Shokryazdan *et al.,* 2017).

The MTT system is used to measure the activity of living cells via mitochondrial dehydrogenases. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt. This exhibits a yellow colour when prepared in media lacking phenol red. Insoluble purple formazan is formed from dissolved MTT by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells.

This water insoluble formazan can be solubilized using DMSO, acidified isopropanol or other solvents (Pure propanol or ethanol). The result of the purple solution was measured spectrophotometrically. The concomitant change in the amount of formazan formed can be visualized by an increase or decrease in the cell number, indicating the degree of effects caused by the test material.

In the present study, *Lactobacillus acidophilus* were isolated from the two different pooled samples, homemade curd and commercial curd and used to investigate their antiproliferation effects against liver cancer (Hep G2) cell line and the effect of the samples on expression of Bax and Bcl 2 gene was analysed by performing RT -PCR.

The main aim and objectives of this study includes that to isolate the probiotic bacterium from homemade curd and commercial curd. Also to determine the cytotoxicity effect of the samples on Hep G2 cell line. And to analyze the gene regulation with Bax and Bcl2 gene for the samples.

Materials and Methods

Collection of samples

Homemade curd and commercial curd were collected from the local outlets of Bengaluru, Karnataka, India.

Media preparation

MRS (De Mann, Rogosa , Sharpe) – 6.16g/L autoclaved at 121^{0} C for 15 min. After incubation, MRS were allowed to cool and poured it into sterile petriplate.

Isolation of bacteria

Samples (100 μL) of homemade curd and commercial curd were serially diluted using sterile phosphate buffer. Serially diluted samples (10^{-2}) and (10^{-3})were placed on MRS agar plates by spread plate technique. The plates were incubated at 37 ^{0} C for 48 hrs and observed for colonies. Bacterial colonies were purified by subsequent subcultures (Sahar Karami *et al.,* 2017).

Antiproliferation assay

Sample preparation

For cytotoxicity studies, organism concentration was maintained to OD 0.1 and cultured in 5 mL MRS broth for 48 hrs. Later organisms were heat killed for 10 min at 80°C. Cells were homogenized thoroughly and were centrifuged at 5000 g for 5 min. Cell free supernatants of *Lactobacillus* strain were collected and two fold dilutions of the broth was carried out using DMEM.

Cell culture

Hep G2 cell lines were procured from American type culture collection (ATCC), stock cells were cultured in DMEM supplemented with 10 % inactivated Fetal

Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) in a humidified atmosphere of 5 % CO_2 at 37 ^oC until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells are checked and centrifuged. Further, 50,000 cells/well was seeded in a 96 well microtitre plate and incubated for 24 hrs at 37^oC in 5 % CO_2 incubator (Maryam Poormontaseri *et al.,* 2017).

MTT assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 5.0 x 10^5 cells/mL using DMEM containing 10 % FBS. To each well of the 96 well microtiter plate, 100 µL of the diluted cell suspension (50,000 cells/well) was added. After 24 hrs, the partial monolayer was formed and the supernatant was flicked off. The monolayer was washed once with medium and 100 µL of different concentrations of samples were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37 ^oC for 24 hrs in 5 % CO_2 atmosphere. After incubation the solutions in the wells were discarded and 100 µL of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 hrs at 37 ^oC in 5 % CO_2 atmosphere. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. Doxorubicin was used as standard. The percentage growth inhibition was calculated using the following formula (Nimmy kumar *et al.,* 2016)

% Inhibition = [(OD of Control – OD of sample)/OD of Control] x 100

Statistical evaluation

IC_{50} Value

The IC_{50} of a sample was determined and the effect examined at different concentrations of samples. IC_{50} values can be calculated by determining the concentration needed to inhibit half of the maximum biological response. IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, SanDiego, CA, USA)

Nonlinear regression

In statistics, nonlinear regression is a form of regression analysis in which observational data are modelled by a function which is a nonlinear combination of the model parameters and depends on one or more independent variables. The data are fitted by a method of successive approximation.

Acridine orange and ethidium bromide dual staining

Dual acridine orange and ethidium bromide (AO/EB) fluorescent staining, visualized under a fluorescent microscope (Kuan Liu *et al.,* 2015). 25 µL (approx. 1x10^5 cells) of treated and untreated cells were taken separately in a micro centrifuge tubes and is stained with 5 µL of AO-EtBr (Acridine orange and Ethidium Bromide) for about 2 min followed by gentle mixing. 10 µL of cell suspension is placed onto a microscopic slide and covered with a glass coverslip and examined in a fluorescence microscope using a fluorescein filter.

Gene regulation using Bax and Bcl2 gene

Extraction of RNA

Total RNA from Hep G2 cells was extracted using TRIzol Reagent (Invitrogen,) according to the manufacturer's instructions. The cells were washed twice with PBS and centrifuged at 2000 g for 5 min. To the cell pellet, 1 mL

of TRIzol was added in 1.5 mL eppendorf tube and vortexed. Samples were maintained at room temperature for 5 min . To the reaction mixture 0.2 mL of chloroform is added and vigorously mixed for 15 seconds. The tube was allowed to stand at room temperature for 5 min, the resulting mixture was centrifuged at 10,000 g for 15 min at 4 ^{0}C. Upper aqueous phase is transferred to a new clean eppendorf tube and treated with 0.5 mL of isopropanol. The resultant mixture is mixed gently by inverting and incubated at room temperature for 5 min. Samples were centrifuged at 10,000 g for 10 min at 4 ^{0}C. Supernatant was discarded and the RNA pellet was treated with by adding 1 mL of 70 % ethanol. The sample was mixed gently by inverting and centrifuged for 5 min at 14,000 g at 4 ^{0}C. Supernatant was discarded by inverting the tube on a clean tissue paper. Later, the pellet was dried by incubating in a dry bath for 5 min at 55 ^{0}C. The pellet was then resuspended in 25 µL of DEPC treated water. The pellet was air dried and dissolved in DEPC treated water (D.Simms *et al.,* 1993)

RT PCR

The RT step is critical for accurate quantification and the amount of cDNA produced must accurately represent RNA input amounts (S A Bustin. 2002). A reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Techno Prime system to determine the levels of Bax and Bcl2 and β-Actin mRNA expressions. The cDNA was synthesized from 2 µg of RNA using the Verso cDNA synthesis kit with oligo dT primer. The reaction volume was set to 20 µL and cDNA synthesis was performed at 42 ^{o}C for 60 min, followed by RT inactivation at 85 ^{o}C for 5 min.

Table.A Primer: details

Gene	Primer pair	Sequence	T_m	Amplicon size (bp)
β – Actin	FP	TCCTCCTGAGCGCAAGTACTCT	62.1	153
	RP	GCTCAGTAACAGTCCGCCTAGAA	62.4	
Bax And Bcl2	FP	GGGAGGTCAGGTGTCCATTG	55.88	152
	RP	TGCTCTCGGGATAGTCACCA	53.83	
	FP	GGTGTCCATTGAGTCACCA	54	
	RP	GCAAGTACTCTCAGTAA	53	

FP : Forward primer ; RP : Reverse primer
Primer source : Erofins primer

PCR

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon) and 1 µM of each complementary primer specific for Bax and Bcl2 and β-Actin (internal control) sequence. The samples were denatured at 94 ^{o}C for 5 min, and amplified using 35 cycles of 94 ^{o}C for 30 seconds each, and for Bax and Bcl2 annealing temperature was set to 49 ^{o}C and for β-Actin the annealing temperature was set to 55 ^{o}C for 30 seconds and elongation at 72 ^{o}C for 1 min followed by a final elongation at 72 ^{o}C for 10 min. The optimal numbers of cycles have been selected for amplification of these genes experimentally so that amplifications were in the exponential range and have not reached a plateau. Ten microliters of the final amplification product

were run on a 2 % ethidium bromide-stained agarose gel and photographed. Quantification of the results were accomplished by measuring the optical density of the bands, using the computerized imaging program Image J. The values were normalized to β-Actin intensity levels.

Results and Discussion

Isolation of bacteria

The bacteria were isolated from homemade and commercial curd and their colonies were counted using colony counter. The results of the bacterial colonies are shown in Table-1.

The Figure 1 and Figure 2 shows the growth of bacteria from homemade and commercial curd respectively.

Antiproliferative effect

Percent of inhibition

The percent of inhibition of the samples homemade and commercial curd on the Hep G2 cell line is tabulated in Table-2.

The results of the MTT assay, in accordance with the percent of inhibition and concentration of the samples denotes that, the percent of inhibition is directly proportional to the concentration of the samples and hence homemade curd shows greater percent of inhibition than that of commercial curd.

Doxorubicin was chosen as a positive control. The proliferation of the cancer cells shall be inhibited by the samples showing lower value for the IC_{50}. Standard Doxorubicin showed an IC_{50} value of 18.69 μM inhibition in Hep G2 cells. Commercial curd and homemade curd showed 54.20 % and 62.44 % inhibition at higher concentration of cell free supernatant.

Acridine orange and ethidium bromide dual staining using HepG2 cell line

Acridine orange stains both live and dead cells whereas Ethidium bromide stains only cells that have lost membrane integrity. Live cells were visualized green. Early apoptotic cells stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells also incorporate Ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin.

Standard doxorubicin 25μM

Dual staining was examined under a fluorescent microscope. Normal cells are seen with circular nucleus uniformly distributed in the center of the cell which is seen in the control Fig 5 and 6. Fig 7, 8, 11 and 12 are showing Early stage apoptotic cells, where cell's nucleus is showing yellow-green fluorescence by Acridine orange (AO) staining and concentrated into a crescent or granular that located in one side of cells.

Staining was localized asymmetrically within the cells. In Fig 9, 10, 13 and 14 it is seen that the nucleus of cells showed orange fluorescence by EtBr staining and gathered in concentration and located in bias. This is late apoptotic phase. Cells that have taken up complete EtBr are the dead cells as in Fig 15 and 16.

The bacteria were isolated from homemade and commercial curd and their colonies were counted using colony counter. The percent of inhibition were calculated on the samples homemade and commercial curd with Hep G2 cell line. Dual staining technique (AO/EtBr)

was done under the fluorescent microscope to visualize the viable cells. Staining was localized asymmetrically within the cells, cells that have taken up complete EtBr are recognized as dead cells. Futher the study was followed for gene regulation by Bax and Bcl2 gene with β-Actin as housekeeping gene. The study concludes that Bcl2 gene showed more fold up regulation when compared with Bax gene on the pooled homemade curd sample.

Table.1 Bacterial colony counts of samples

Sample	Dilutions	Colony counts (CFU/g)
Homemade Curd	10^{-2}	267
	10^{-3}	158
Commercial Curd	10^{-2}	537
	10^{-3}	341

Table.2 Percent of inhibition of curd samples

Hep G2 cell line			
Samples	**Conc. in %**	**OD at 590nm**	**% Inhibition**
Control	0	0.583	0.00
Commercial curd	1.56	0.510	12.52
	3.13	0.458	21.44
	6.25	0.376	35.49
	12.50	0.341	41.51
	25.00	0.304	47.86
	50.00	0.267	54.20
Homemade curd	1.56	0.502	13.83
	3.13	0.432	25.90
	6.25	0.380	34.82
	12.50	0.297	49.06
	25.00	0.253	56.60
	50.00	0.219	62.44

Table.3 IC_{50} of the *Lactobacillus* strain supernatants on Hep G2 cell line

Hep G2 cell line				
Samples	**Conc. µM**	**OD at 590nm**	**% Inhibition**	**IC_{50} µM**
Control	0	0.583	0.00	**18.69**
Doxorubicin	3.13	0.403	30.82	
	6.25	0.366	37.16	
	12.5	0.294	49.57	
	25	0.223	61.67	
	50	0.176	69.73	
	100	0.111	80.89	

Table.4 Relative expression of Bax and Bcl2 gene in homemade curd

Gene	Samples (µL)	Band Intensity Of PCR Amplicon Of Genes		Normalised	Relative Gene Expression
		β-Actin	Bax and Bcl2		
Bax	**25**	21786.57	4915.2	0.226	0.210
	12.5	20556.18	16778.76	0.816	0.759
	Control	16661.52	17919.18	1.075	1
Bcl2	**Control**	21380.69	16078.93	0.752	1
	12	17729.28	11083.71	0.625	0.831
	25	16323.66	5469.08	0.335	0.446

Table.5 Relative expression of Bax and Bcl2 gene in commercial curd

Gene	Samples (µL)	Band Intensity Of PCR Amplicon Of Genes		Normalised	Relative Gene Expression
		β-Actin	Bax and Bcl2		
Bax	50	14189.40	6194.32	0.44	0.409
	25	15784.27	8547.76	0.54	0.507
	Control	16867.00	18023.49	1.07	1
Bcl2	Control	14266.15	15215.47	1.07	1
	25	15280.23	11787.57	0.77	0.723
	50	14229.38	8705.61	0.61	0.574

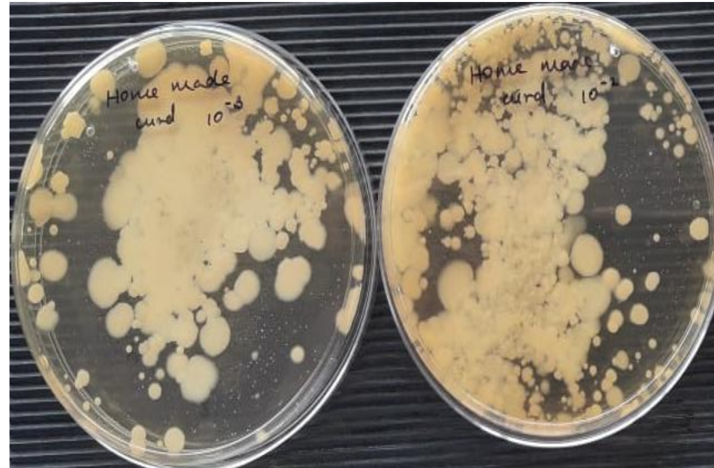

Fig.1 Homemade Curd plating of 10^{-2} and 10^{-3}

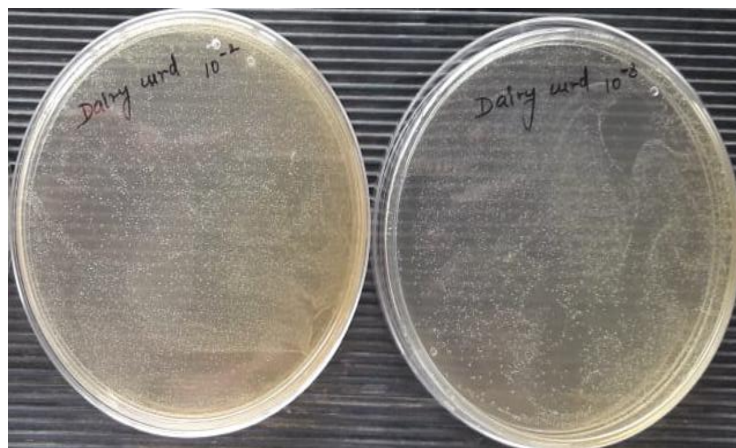

Fig.2 Commercial curd plating of 10^{-2} and 10^{-3}

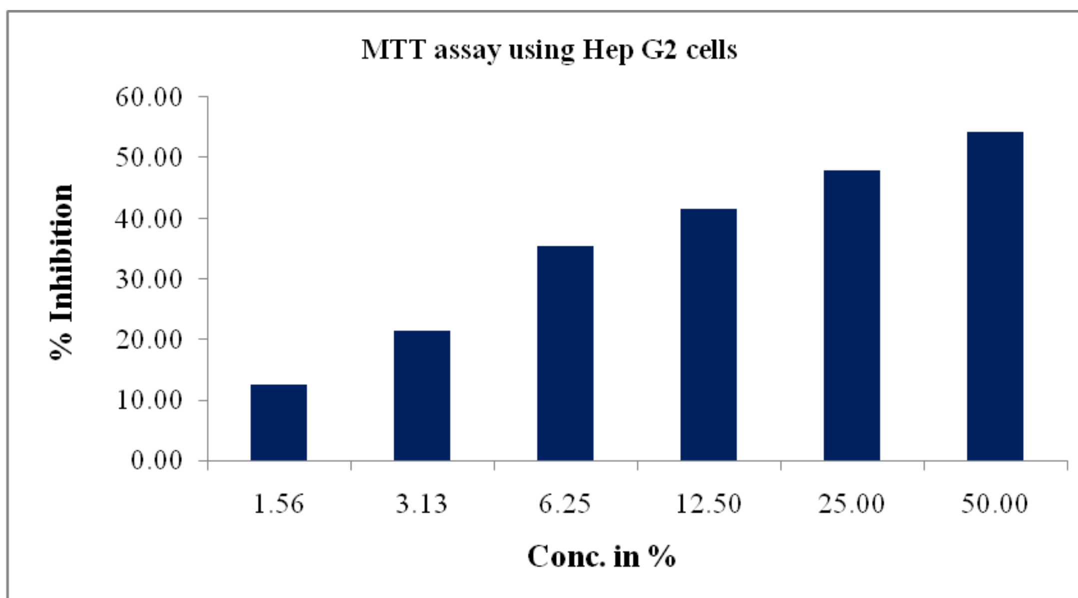

Figure.3 Bar graph for percent of inhibition

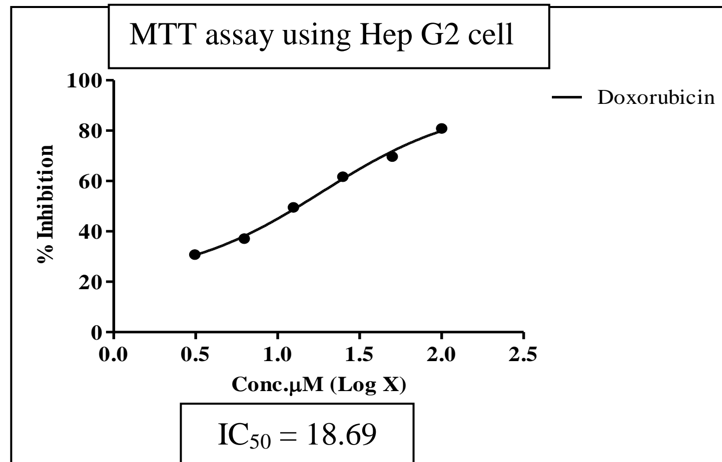

Figure.4 Percentage inhibition of doxorubicin

Control HepG2 cells

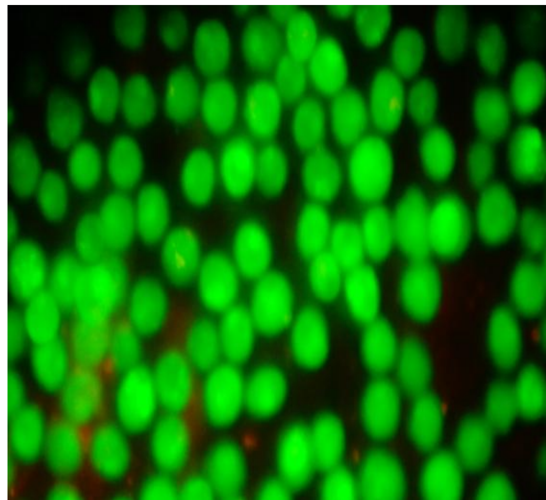

Figure.5 Normal cells - treated cells

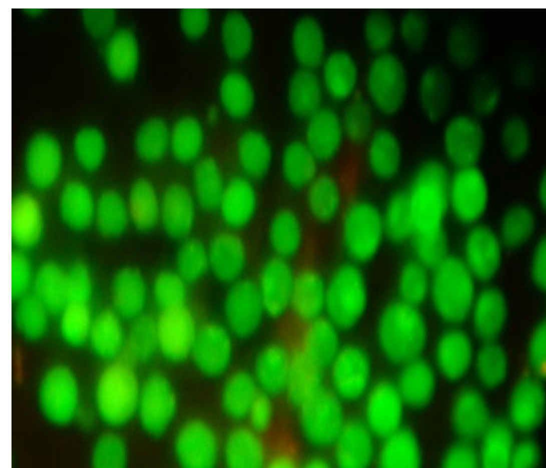

Figure.6 Normal cells - untreated cells

Conc. 12.5% of homemade curd

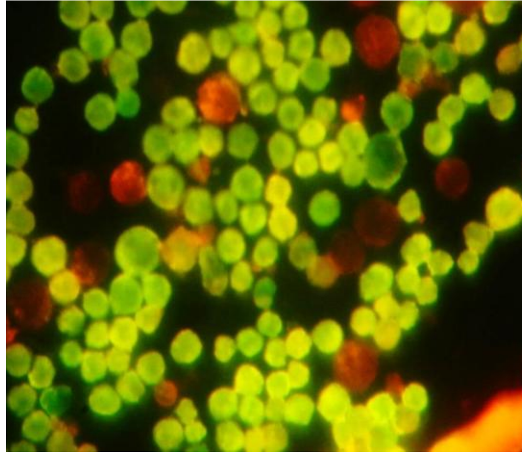

Figure.7 Early stage apoptotic phase - treated cells

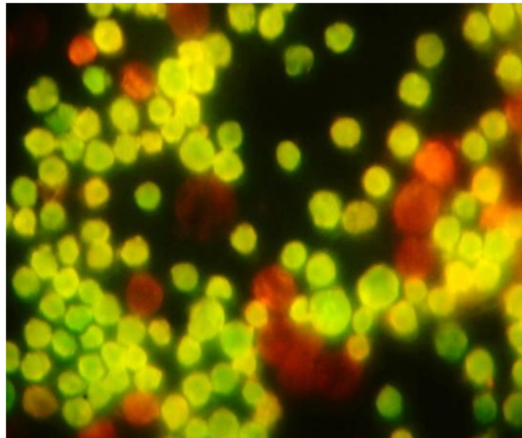

Figure.8 Early stage apoptotic phase - untreated cells

Conc. 25% of homemade curd

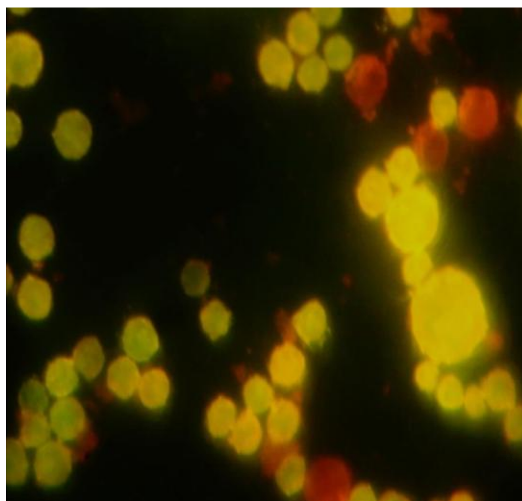

Figure.9 Late stage apoptotic phase -treated cells

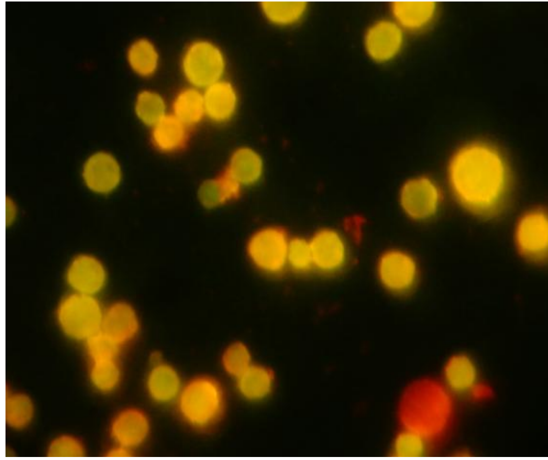

Figure.10 Late apoptotic phase - untreated cells

Conc. 25% of commercial curd

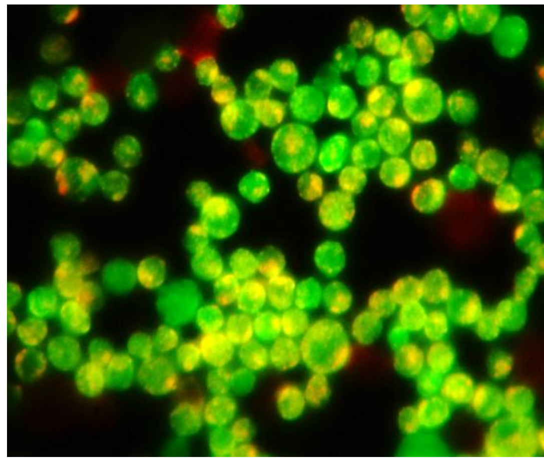

Figure.11 Early apoptotic cells - treated cells

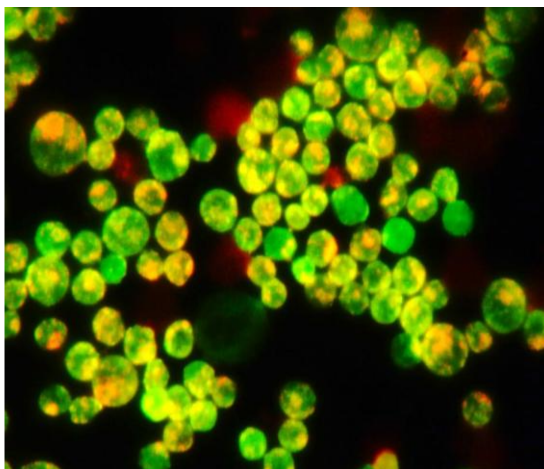

Figure.12 Early stage apoptotic cell - untreated cells

Conc. 50% of commercial curd

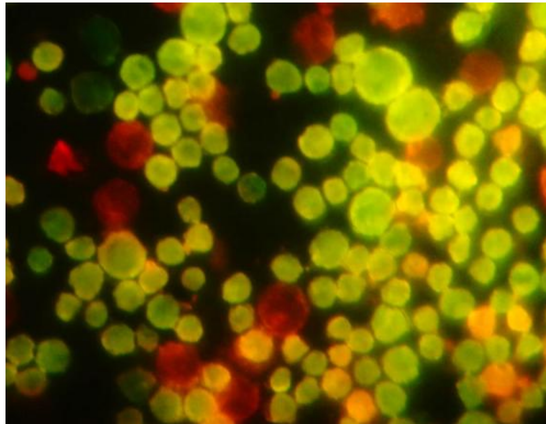

Figure.13 Late stage apoptotic phase - treated cells

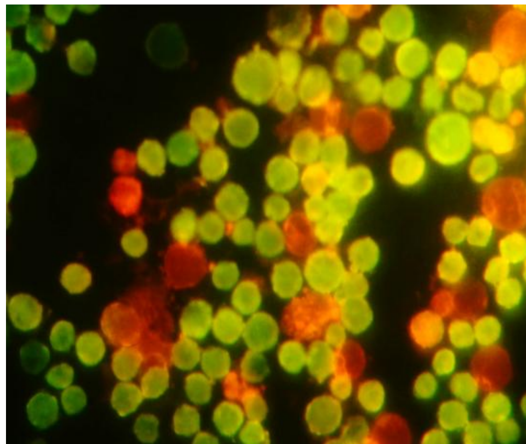

Figure.14 Late stage apoptotic phase - untreated cells

Standard Doxorubicin 25µM

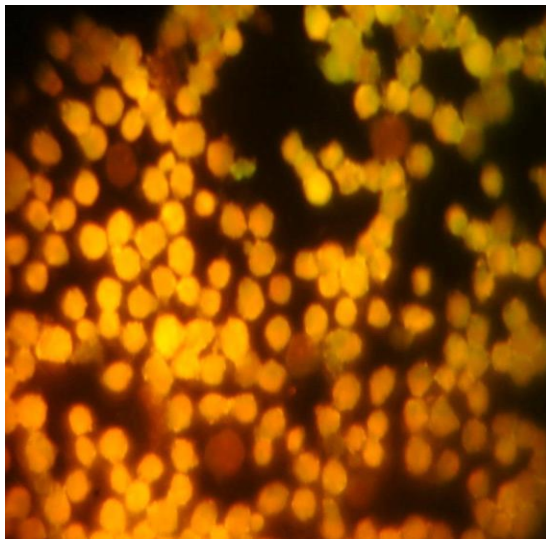

Figure.15 Dead cells - treated cells

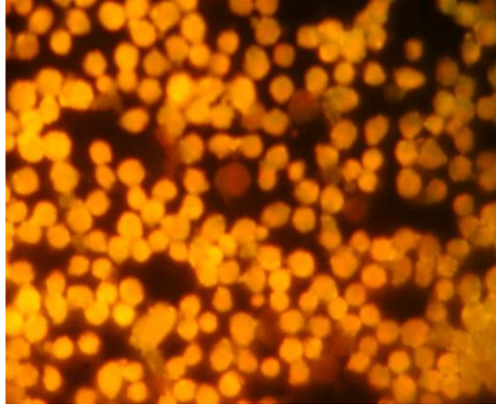

Figure.16 Dead cells - untreated cells

Comparison of amplicons - Amplicons of homemade curd

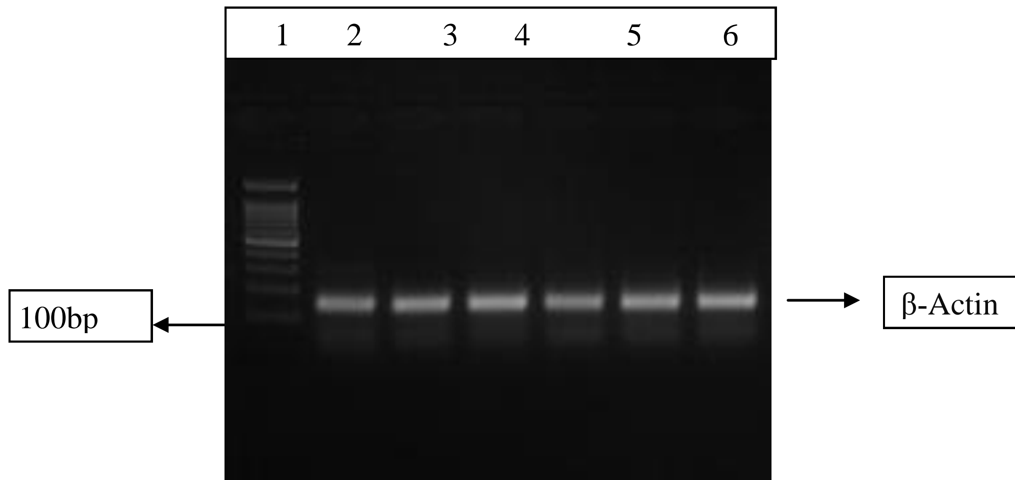

Figure.17 Amplification of β-Actin gene in Hep G2 cell line

Lane 1- DNA Ladder ; Lane 2– 500 µL; Lane 3- 250 µL; Lane 4- Control; Lane 5- Control, Lane 6-250 µL, Lane 7- 500 µL, Lane 2,3,6,7 - Samples with β-Actin gene

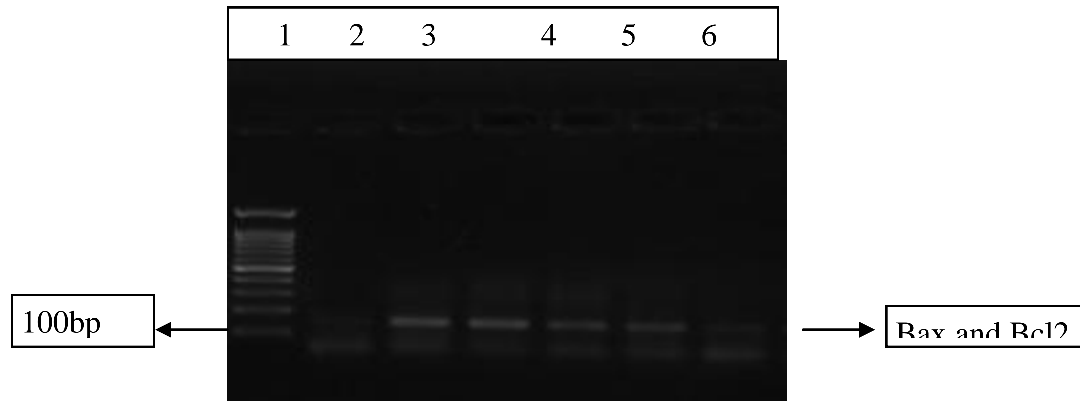

Figure.18 Amplification of Bax and Bcl 2 gene in Hep G2 cell line

Lane 1- DNA Ladder ; Lane 2–500 µL; Lane 3-250 µL; Lane 4- Control; Lane 5- Control, Lane 6-250 µL, Lane 7- 500 µL, Lane 2,3 - Samples with Bax gene ; Lane 6,7 - Samples with Bcl2 gene

Amplicons of commercial curd

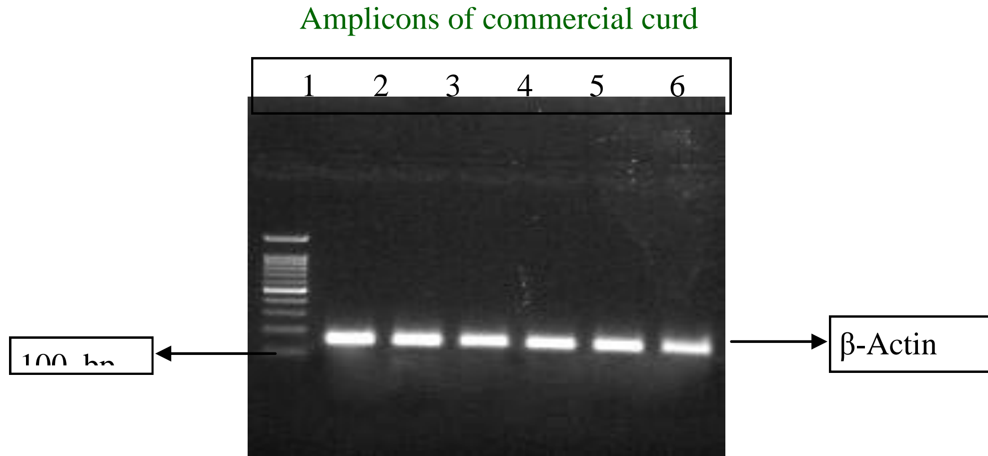

Figure.19 Amplification of β-Actin gene in Hep G2 Cell line
Lane 1- DNA Ladder ; Lane 2– 500 µL; Lane 3-250 µL; Lane 4- Control; Lane 5- Control, Lane 6-250 µL, Lane 7- 500 µL, Lane 2,3,6,7 - Samples with β-Actin gene

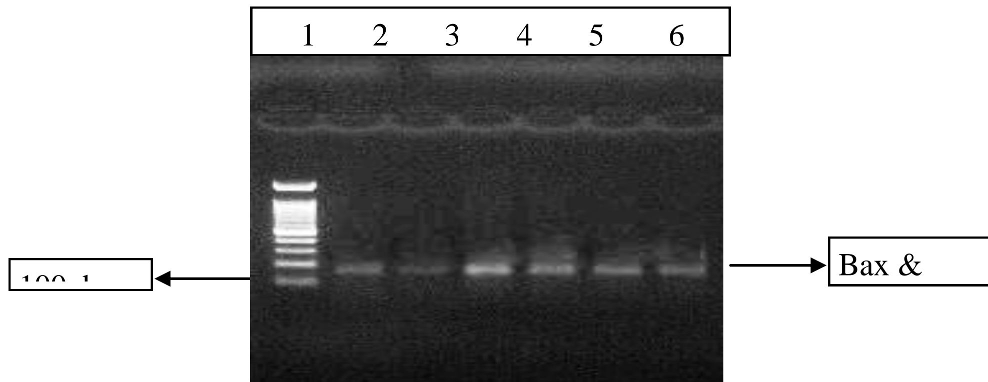

Figure.20 Amplification of Bax and Bcl2 gene in Hep G2 Cell line
Lane 1- DNA Ladder ; Lane 2– 500 µL; Lane 3-250 µL; Lane 4- Control; Lane 5- Control, Lane 6-250 µL, Lane 7- 500 µL, Lane 2,3 - Samples with Bax gene ; Lane 6,7 - Samples with Bcl2 gene

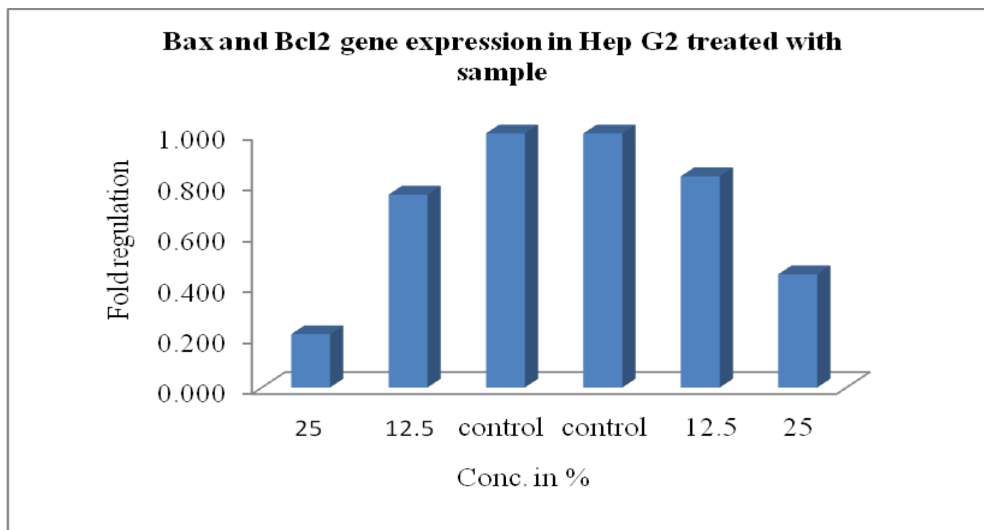

Figure.21 Fold regulation of Bax and Bcl2 gene in homemade curd

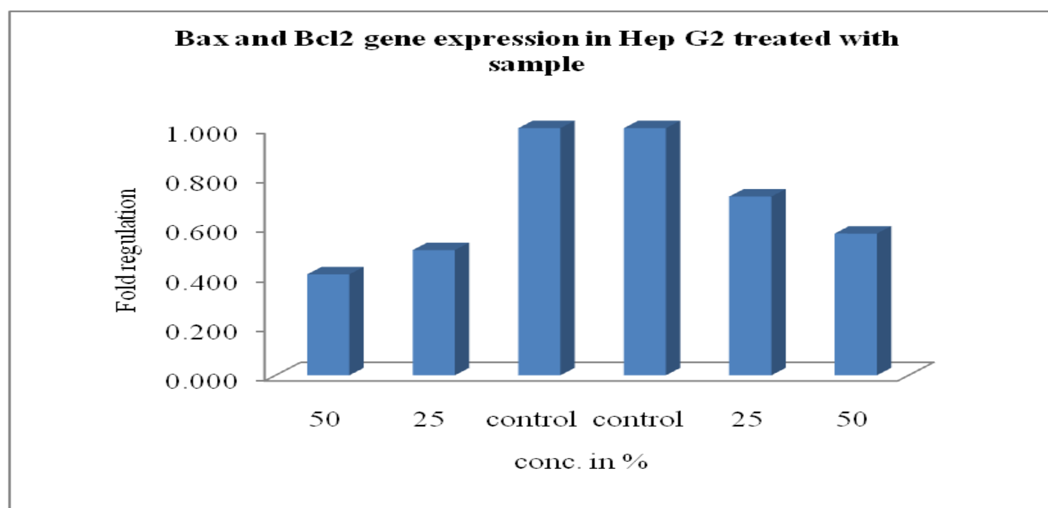

Figure.22 Relative expression of Bax and Bcl2 gene in commercial curd

In conclusion, our study disclosed that the probiotic bacteria was isolated from two different sources such as homemade curd and commercial curd and was analyzed for their antiproliferative activity by MTT assay on Hep G2 cell line. Based on the IC_{50} value, homemade curd shows more percent of inhibition than commercial curd. In this study, effect of Homemade curd and commercial curd for expression of Bax and Bcl2 gene was studied in Hep G2 cell by RT-PCR. The internal control β-Actin was used to normalize the gene expression. Bcl2 showed fold up regulation when compared to Bax. The results of this study revealed that the MTT assay for the probiotic bacterium isolated from the sample has better cytotoxicity effect.

Acknowledgement

We are thankful to Skanda Life Science Pvt.Ltd. Bengaluru, for providing us the laboratory facilities to carry out this project.

References

Bustin, S A. 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, 29:23-39.

Dicks LMT and Botes M, 2010. Probiotic lactic acid bacteria in gastro-intestinal tract: health benefits, safety and mode of action. Beneficial Microbes, 1: 11–29.

Kuan Liu, Peng-cheng Liu, Run Liu, Xing Wu. 2015 Dual AO/ED staining to detect apoptosis in osteosarcoma cells compared with flow cytometry, 21:15-20.

Maryam Poormontaseri, Saeid Hosseinzadeh, Seyed Shahram Shekarforoush, and Tahereh Kalantari, 2017. The effects of probiotic *Bacillus subtilis* on the cytotoxicity of *Clostridium perfringens* type a in Caco-2 cell culture, 17: 150.

Nimmy Kumar, Subhankar Biswas, Asha Elizabeth Mathew, Subin Varhese, Jessy Elizabeth, K. Nada Kumar, Jesil Mathew Arajani, Richard Lobo. 2016 Pro apoptotic and cytotoxic effect of enriched fraction of Elytranthe parasitica (L). Danser against HepG2 Hepatocellular carcinoma, 16:420.

Parisa Shokryazdan, Mohammad Faseleh Jahromi, Fatemeh Bashokouh, Zulkifli Idrus, Juan Boo Liang, 2017. Antiproliferation effects and antioxidant activity of two

new *Lactobacillus* strains, 21

Sahar Karami, Mohammad Roayaei, Hosna Hamzavi, Mahmoud Bahmani, Hassan Hassanzad-Azar, Mahmoodnia Leila, Mahmoud Rafieian-Kopaei, 2017 Isolation and identification of probiotic Lactobacillus from local dairy and evaluating their antagonistic effect on pathogens, 7:137-41.

Simms, D., PE Cizdzial, P Chomczynki. 1993 TRIZOL :A new reagent for optimal single step isolation of RNA, 4:99-102.

How to cite this article:

Ramesh Babu. N. G., K. A. Simrah Fathima, V. Nandhini and Nandhini. V. 2020. Cytotoxicity Effects of *Lactobacillus Acidophilus* on Hep G2 Cell Line and its Effects on Gene Regulation with Bax & Bcl2. *Int.J.Curr.Microbiol.App.Sci.* 9(05): 891-906.
doi: https://doi.org/10.20546/ijcmas.2020.905.098