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Cytotoxicity potential of local isolates of *Lactobacillus Acidophilus* Extracts on Colon Cancer Cell Line

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ABSTRACT

Lactobacillus acidophilus isolated locally from dairy samples and identified by biochemical and molecular methods bacterial extract were examined to explore potential antitumor activity by apply on colon cancer cell line CaCo-2.The viability results revealed that all strains extract have cytotoxicity effect on colon cancer cell line in ranged between 36-79% at 400 µg/ml,and more affected one was chosen for more analysis by high content screening assays that show the extract decreased significantly the viability in concentrations ranged between 200-25 100µg/ml.mitochondrial membrane potential and cytochrome C releasing increased significantly as results of apoptosis processes ,while membrane permeability did not affected decreased slightly result of necrosis process.

Keywords

Lactobacillus acidophilus; Antitumor activity; cancer cell line; cytotoxicity effect

Introduction

A great attention was paid to the use of microorganisms or their metabolites in the field of industry, food safety and in the treatment of some diseases. Bacteria are the first type of microorganisms used in this approach. Among the bacterial groups is the Lactobacillus spp., which have a great role in probiotics[1]. Bacterial researches focused more on commercial aspects and possible health application. This interest is due to the range of possible health effects of these bacteria, which possess potential therapeutic properties including anti-inflammatory and anti-cancer activities, as well as other features of interest like degradation of aflatoxin B1 (1,2,3) . Cancer is consider one of the main cause of death worldwide [1]. Cancer cells could escape normal growth process . in normal conditions, there is a mechanism regulate the growth and death in cells, so the number of specified cell kinds remains stable. tumor cells are featured by different of cellular biochemical alterations, including: self-sufficiency of growth chemical signals, unrespond to inhibitors signals and apoptosis processes , unlimited replication, continuous metastasis [2].

Lactobacillus have anti-proliferation, apoptosis and anticancer properties in some cancer cell lines [7,10]. Previous study showed that the ferrous pigment extracted from Lactobacillus showed a tumor suppressor effect via the JNK signaling pathway [11,12]. The foregoing researches have shown that specific compounds produced by lactic acid bacteria can lead to anti-tumor molecules to suppresor cancer cells [8,9,13].

Available cancer therapy protocols include chemotherapy, surgery, and treatment by radiation therapy. In most cases, chemotherapy is the main treatment option. However, most conventional chemotherapeutics that target active ells in divission without efficient selectivity towards cancer cells and that lead to high damage on normal cells also. In addition, tumor cells could protect it self from chemotherapy by different mechanisms, such as production of drug detoxification enzymes and drug transporters, and improving the capability to fix DNA damage in cell mechanisms that mediate program cell death [3]. So, there is an urgent need for therapy that can selectively kill cancer cells as an adjuvant to reduce the drug dose and improve the efficacy of anti-cancer drugs without affecting normal healthy cells. Lactic acid bacteria (LAB) synthesis of extracellular polysaccharide is a well-known phenomenon, which exists in the form of cell-bound EPS, which adheres tightly to the surface of bacteria, or releases lipopolysaccarides into the medium (4).

Lactobacillus species are famous bacteria used to synthesize exopolysaccharides. It begin from the EPS bind by cells attached to the surface of the bacteria, and then is released into the surrounding medium. It is believed that have an important function in the stabilization of lactobacillus in the small intestinal mucosa [4, 5]. Some lactic acid bacteria produced active compounds not only have the capability to induce immunity and antioxidant properties, but also have anti-tumor and anti-biofilm effects. They have many health benefits and have attracted special attention, [6] reported a method of extracting and purifying EPS from Lactobacillus plantarum YML009, which has strong scavenging activity. And anticancer potential on liver cancer cell lines (HepG-2) [7].

In this study, we examined Lactobacillus acidophilus filtrate and evaluated its selective cytotoxicity to human colon cancer cell line. In addition, to the possible mechanism of the anti-cancer activity of the extract has been studied at the cellular and molecular levels.

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Materials and Methods

Bacterial Isolates

Lactobacillus strains were isolated from samples of home made yoghurt from cow milk. Yoghurt samples were transported in MRS broth incubated for 48 hours then were inoculated onto MRS agar pH 5.2 and incubated at 37°C for 48 hours, then inoculated onto MRS agar pH 4.3 at 37°C in anaerobic jar for 48 hours . Single colonies were then taken from MRS agar and cultured, each on MRS agar, then identified as L. acidophilus and used in this study.

Identification of Lactobacillus acidophilus

Microscope Examination

Part of isolate colony was fixed on a microscopic slide, then stained by Gram stain to examine cells shape, grouping, gram reaction and spore forming (13).

Biochemical Tests

Catalase test : This test was performed by adding few drops of H2O2 (3%) onto single bacterial colony on a clean slide. Production of gaseous bubbles indicates the ability of bacteria to produce catalase enzyme (positive result) . Oxidase test Single bacterial colony from bacterial growth was picked up and smeared on filter paper moistened with a few drops of an oxidase reagent. A positive reaction is determined via deep purple colour appearing within few seconds,(13).

Carbohydrate fermentation

This assay done by API CH 50 (Biomeruex) is a standardized system associating 50 biochemical test for the study of the carbohydrate metabolism of microorganism.

Molecular Identification of Lactobacillus acidophilus by PCR

Bacterial DNA were extracted according to manfacure compant protocol of Bioneer kit.The purified DNA from the samples was diluted with TE buffer to 1:100 and the DNA concentration and purity were determined spectrphotometrically by measuring their absorbance at 260 (A_{260}) and 280 (A_{280}), as advised by Maniats *et al*. (1989). A PCR technique was used with primers specified *Lactobacillus acidophilus* bacterial DNA that located between 16S and 23S ribosomal RNA (14).

The lyophilized primers were diluted in 1 ml of TE (pH 8.0) and kept as a stock in -20ºC .Then 100 µl of this stock was diluted in 1ml of deionized sterile distilled water to obtain nearly 10 picomoles/µl.

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A master mix of PCR)12.5 µl(was mixed with(3 µl) bacterial DNA and (1 µl) from each designed primer forward and reverse, then completed volume up to 25 µl with free nucleases deionized distilled water according to the instructions of the company.

Primers name	Sequences (5'-3')	Predictive product
Laci-1(Forward)	TGCAAAGTGGTAGCGTAAGC	
23-10C(Reverse)	CCTTTCCCTCACGGTACTG	

Preparation of Lactobacillus acidophilus extract

Inoculated 10^8 CFU/ml of *L. acidophilus* into 100 ml of MRS broth medium , incubated for 72 hour at 37 ˚C in 5% CO_2 . Growth culture was sonicated at 120 MHZ, for 120 minutes ,then Growth culture was filtrated by Millipore filter (0.45µ). The filtrate was dried by lyophilizer and dry extract was kept at -20 ˚C until being used .

MTT Cell Assay

Six strains of *L.acidophilus* extracts were tested for their cytotoxicity properties on CaCo2 cell line by mtt assay using MTT (interon Ltd kit).The extract concentrations was 400,200,100,50,25µg/ml. cultured for 72h. on RPMI1640 medium, then Cells were detached by a trypsin /EDTA solution. Cells suspended in medium containing 0.1% bovine serum albumin ,and seeded in 96 –well microtiter plates, then tested for their antitumor potential activity by the MTT assay according to the manufacture protocol and read later on 517 nm.

Apoptosis Assay

High content screening (thermofisher scientific company) was used to determine apoptosis potential in concentration 100,50,25.12.5 and 6.25µg/ml by measuring Viability, membrane potential, mitochondrial membrane potential, nuclear intensity and cytochrome C releasing.HCS is a powerful tools employs fluorescence indicators to define cellular morphology and molecular response to compounds treatment . High screening system was done in pharmacology department medicine college in Malaya university in Malaysia .

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Results and discussion

Morphological Characteristics

Colonies of LAB grown on MRS agar medium appears white to pale in color, 2-5 mm in diameter, round shape, soft, mucoid, convex and having smooth edges.

A microscopical examination of these isolates show that they are Gram positive, bacilli, clustered in short and long chains containing (3-8) cells, and rarely single. They are also non-spore forming and non-motile.

Biochemical Tests

Many Biochemical tests were carried out to identify the isolate used in this study ,Results showed that the isolate gave negative results for catalase due to the inability to produce catalase enzyme (that reduce hydrogen peroxide to water and oxygen gas bubbles), negative results for oxidase due to the inability to produce cytochrome oxidase (that oxidase tetramethyl-p-phenylenediamine.These results were in agreement with those observed by (14,15).

Carbohydrate fermentation

A carbohydrate fermentation profile was obtained by API CH 50 strips (Biomeruex) used to identify the isolates of *Lactobacillus*, in duplicate, at 37 ˚C in *Lactobacillus* medium(CHL broth of API 50). The outcome showed that *Lactobacillus* isolate fermented the sugars (Galactose, D-Glucose , D-Fructose , D-Mannose , N-Acetyl-glucosamine, Amygdalin , Arbutin , Esculin , Salicin , Cellobiose D-,Maltose,Lactose , Saccharose , Trehalose , D,Raffinose , Amidon , Glycogen ,β-Gentiobiose), which gave a positive result .

On the othe hand, the sugars (D-Arabinose , L- Arabinose , Ribose , D-Xylose , L-Xylose , Adonitol 2-methyl-, Gluconate ,2-Keto-gluconate, 5-keto-gluconate , β-Methyl-D-xiloside , L-Sorbose , Rhamnose , Dulcitol , Inositol , Mannitol , Sorbitol , α-Methyl-D-mannoside , α-Methyl-D-glucoside , Melibiose , Inulin, Melezitose , Xylitol , β-Gentiobiose , D-Arabitol , D-Lyxose , D-Tagatose , D-Fucose , L-Fucose , CTRL ,Glycerol , Erthritol ,D-Turanose , L-Arabitol ,)were not fermented by these bacteria and gave a negative result. (Table 4.2 and figure 4.1) This confirmed that the isolate is *Lactobacillus acidophilus* .

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Molecular identification of Lactobacillus acidophilus

A molecular diagnosis for isolates was carried out using PCR analysis to confirm our diagnosis mentioned above .Specific primers were used according to (16). Recently, the use of genetic analyses was represented an advance in the taxonomy of lactobacilli . Genotype-based identification methods solved the problem of variable phenotype to provide more accurate species identification(17).The use of specific sequences of bacterial DNA ,which codify the intergenic spacer region between 16S and 23S ribosomal RNA , in diagnosis ,was because its sequence is stable and has long been used as a taxonomic "gold standard" in determining the phylogenies of bacterial species(17,18).

The molecular identification result clarified that the isolate belonged to *L. acidophilus* because the amplified fragment appeared as 210 pb, as shown in figure (1) and that is in agreement with which mentioned by (18).

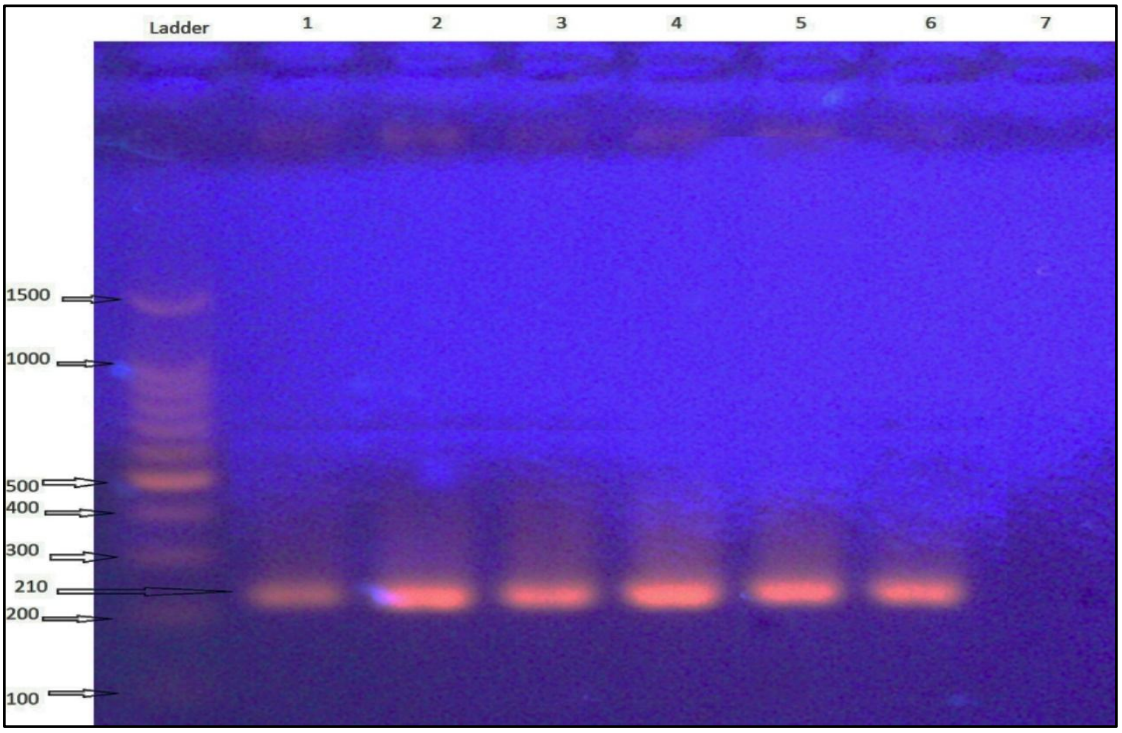

Figure 1. Detection of PCR product DNA bands of *Lactobacillus acidophilus* from (1 to 6) positive ,(7) Negative.

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Antitumor effect of Lactobacillus acidophilus on cell line

Cytotoxicity assay

3-(dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide (MTT) stain was used to detect the cytotoxic effect of six strains from *L. acidophilus* on colon cancer cell line CaCo-2. This assay was performed to measure the cell proliferation and inhibition rate by using different concentrations of *L. acidophilus* growth extracts on CaCo-2 cell line.

The extracts of strains LB3 and LB5 have high cytotoxicity on colon cancer cell line with IC50 40.8 and 51µg/ml respectively as in figure 2 and 3.while other strains showed weak or moderate cytotoxicity and the inhibition rate for all other strain extracts ranged between 49-21% at concentration 400 µg/ml as in table.1.

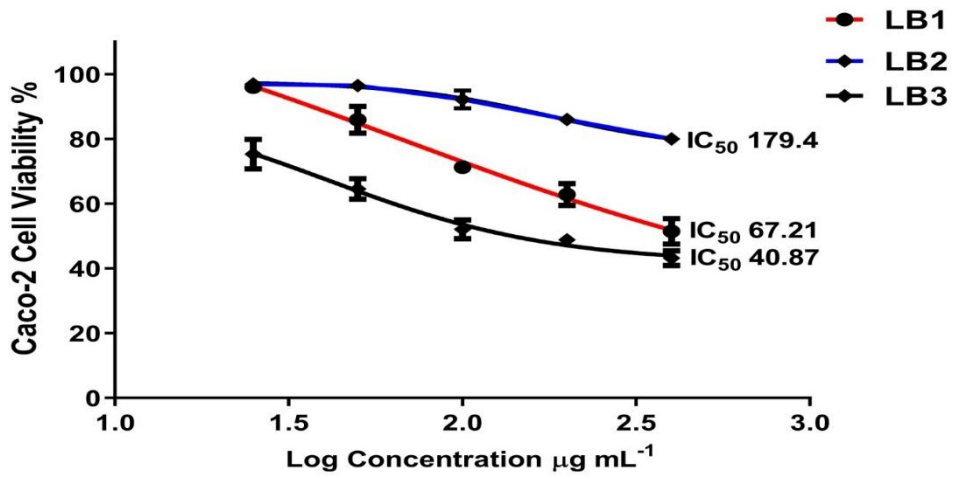

Figure 2. Cytotoxicity of *L.acidophilus* B1,B2,B3 extracts on the growth of CaCo-2 tumor cells

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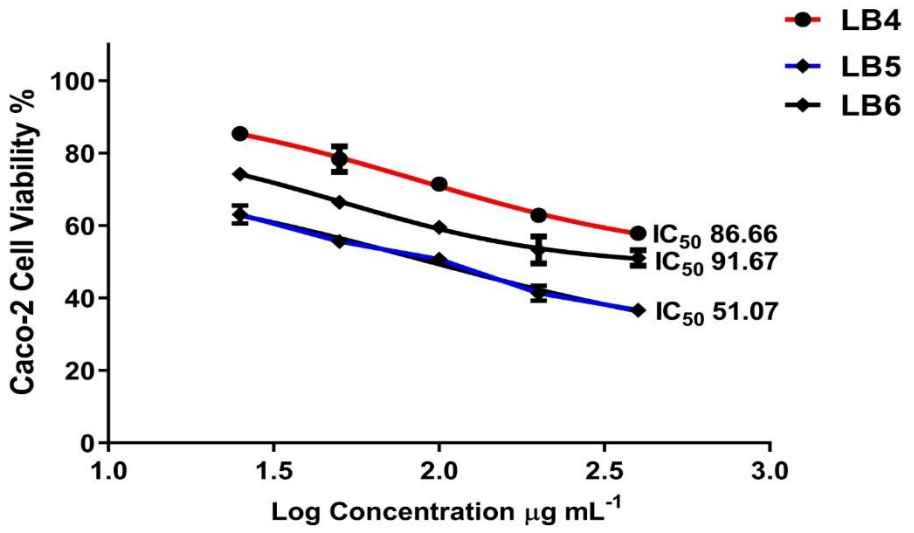

Figure3.

Cytotoxicity of *L.acidophilus* B4,B5,B6 extracts on the growth of CaCo-2 tumor cells

Table 1.viability rate of Colon cancer cell line subjected to extracts of different *L.acidophillus* strains.

Viability%(mean±SD)					
µg/ml Strains	**400**	**200**	**100**	**50**	**25**
LB1	51.4±3.9	62.8±3.4	71.2±1.5	85.9±4.1	95.9±0.8
LB2	79.9±1.8	86±1.9	92.2±2.7	96.4±1.2	96.5±1.1
LB3	43.1±2.2	48.4±0.2	52±2.9	64.5±3.1	75.3±4.5
LB4	57.8±0.9	62.8±1.9	71.4±0.7	78.3±3.53	85.3±1.1
LB5	36.6±1.1	41.3±2	50.6±1.7	55.5±1.6	63±2.5
LB6	51±2.1	53.2±3.7	59.2±1.4	66.4±0.7	74.1±1.8

Numerous studies was determined the cytotoxicity of *L. acidophilus* extracts on different cell line [22] mentioned that the S-layer protein filtrate of *L. acidophilus* have different effect on human Rhabdomyosarcoma (RD) cell line and Recombinant murine (L20B) cell line depends on their concentration. Lactic acid bacteria could decrease genotoxicity of dietary carcinogens *in*

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vitro depending on bacterial species strongly [20].[21] examined antitumour of 4 lactic acid producing bacteria strains, (*Bifidobacterium animalis* , *lactobacillus delbreukii* , *lactobacillus plantarum* and *lactobacillus acidophilus*)on four human cancer cell – lines (HEPG-2 , MCF-7, Henrietta Lacks cervix carcinoma (HELA) and colon carcinoma (HCTI16) , they found that maximum inhibition was exerted by *B.animalis* on four cell lines which were showed different affect by *L. acidophilus*.

Apoptosis Assay

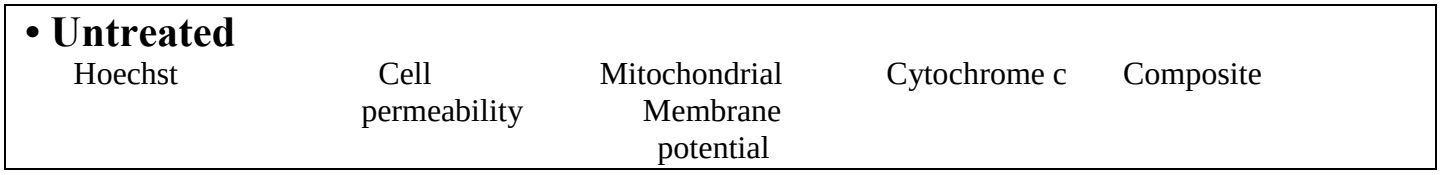
High content screening was done on the CaCo-2 cell line (fig. 4) , to more clarify the cell-health parameters (nuclear morphology cell membrane permeability, mitochondrial membrane potential changes and cytochrome c localization and release from mitochondria).

Theresults revealed that there was decrease in viability rate about 42% in comparing with control at 200µg/ml.(figure.5)

The differences in these results of viability in compared with MTT assays is that in HSC the data express effect of early exposure to the drug and its designs basically and for detection morphological and cellular daises in cells through few hours of treatment while in MTT the assay cells combined with drug for longer time.

Also the degradation od DNA was notice by increasing total nuclear intensity in significant way at 200 and 10e0 µg/ml. of extract (Figure .6).Apoptosis processes indicated by decreasing mitochondrial membrane potential in significant manner at concentrations 200 and 100 µg/ml,and the membrane permeability decreasing 37% in comparing with control and that lead to increase cytochrome C significantly at 200 and 100 µg/ml.as in (figures 7,8).

Membrane permeability did not have certain effect unless at high concentrations ,200 µg/ml from extract, while other concentrations did not differ significantly from the control(Figure.9).



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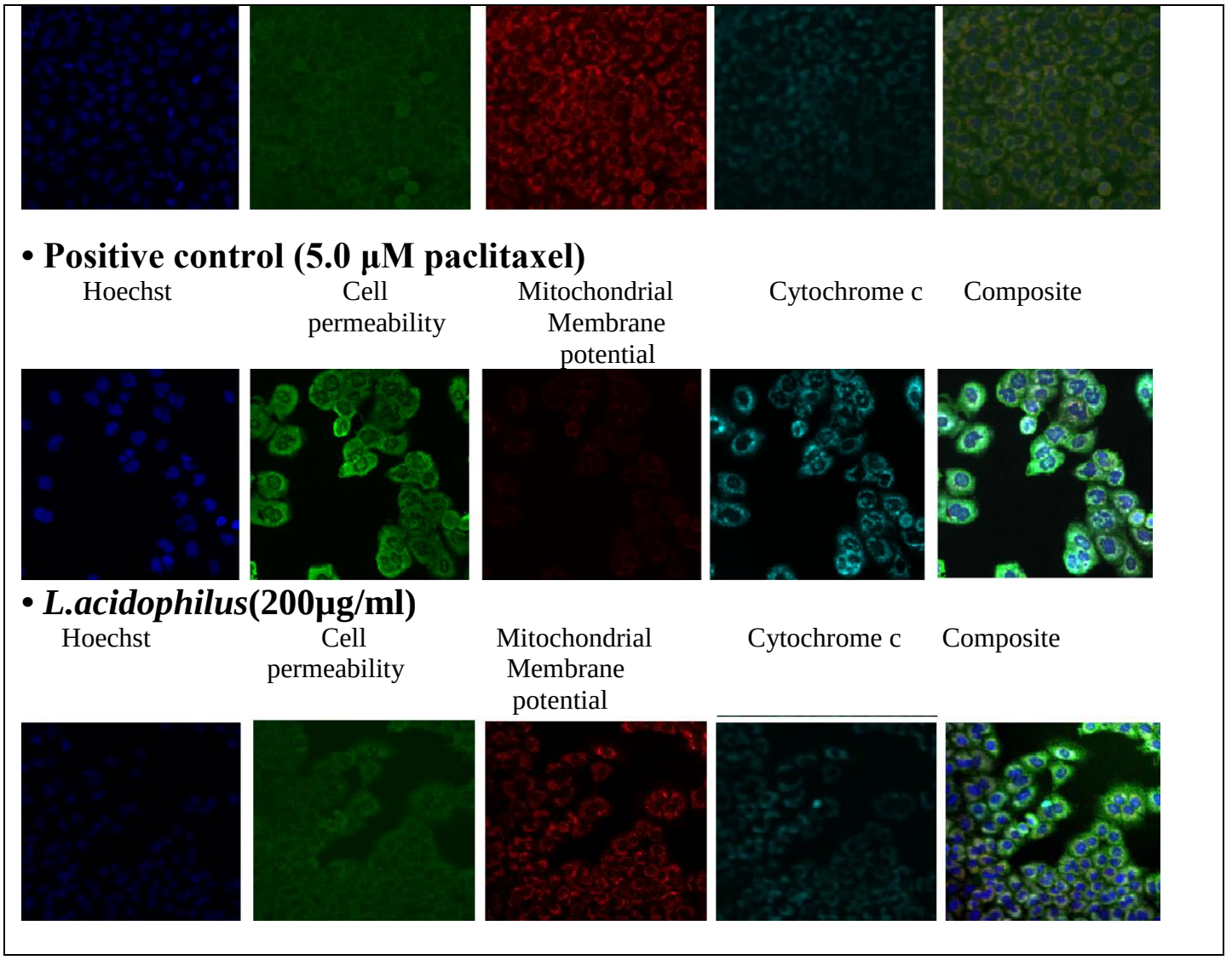

Figure .4. High screening content on CaCo-2 cells

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Valid cell counts:

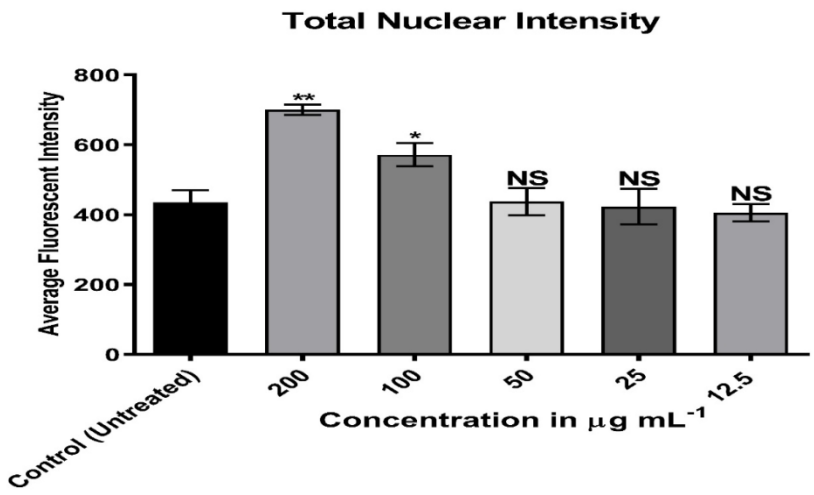

Figure 5. Effect of *L.acidophilus* extracts concentration on Valid CaC0-2 tumor cells counts

Total Nuclear Intensity

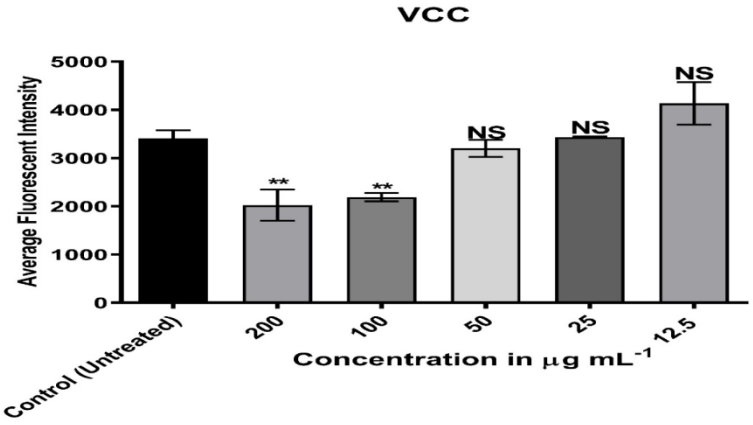

Figure.6. Effect of *L.acidophilus* extracts concentration on Total nuclear intensity of CaC0-2 tumor cells

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Mitochondrial membrane potential

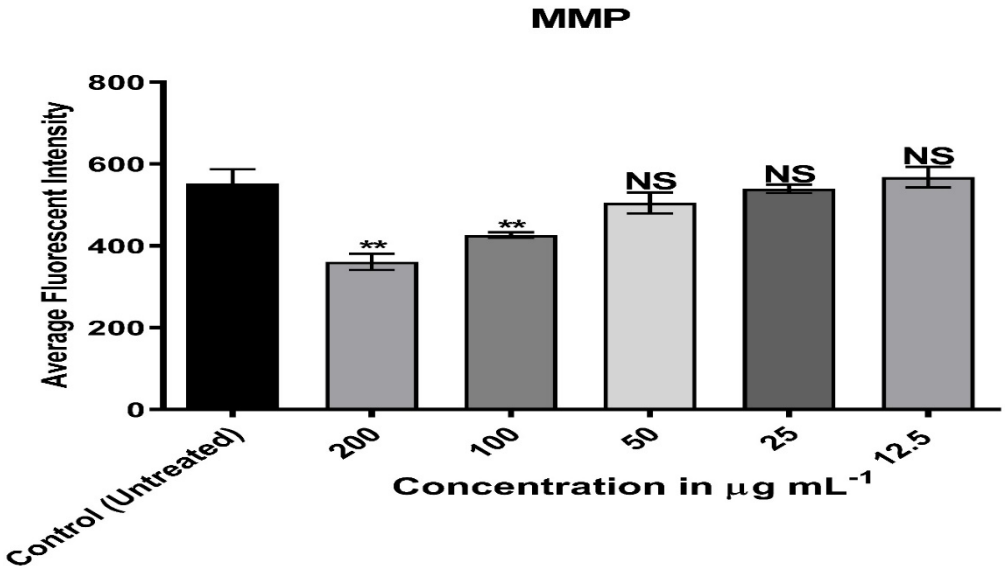

Figure.7. Effect of L.acidophilus extracts concentration on CaC0-2 tumor cells Mitochondrial membrane potential

Cytochroec

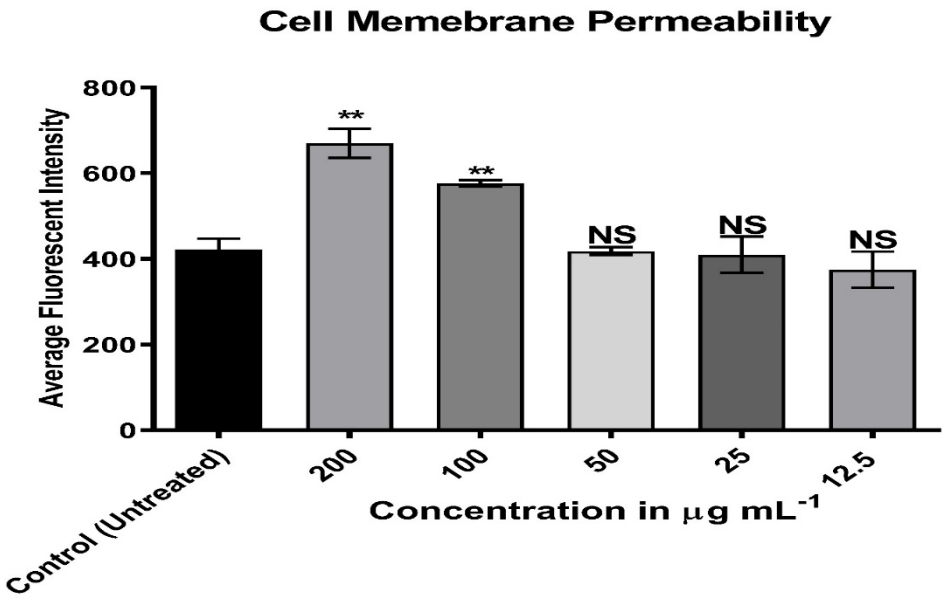

Figure 8. Effect of *L.acidophilus* extracts concentration on Cytochrome C of CaC0-2 tumor cells.

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Cell Membrane Permeability

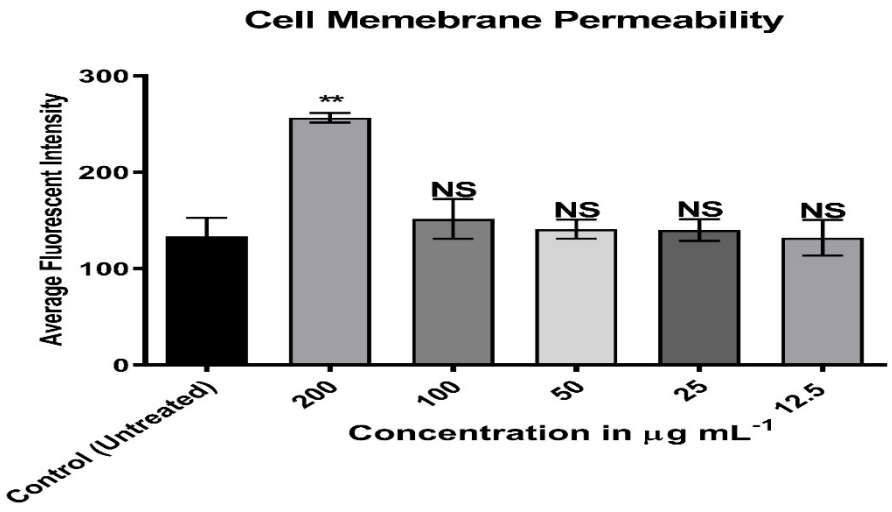

Figure.9. Effect of L.acidophilus extracts concentration on CaC0-2 tumor cells membrane permeability**.**

research has focused on potential of beneficial bacteria to inhibit tumor development. The researches that included *Lactobacillus ac*idophillus as apoptosis inducer is still limited. Earlier researches have revealed that the protection effect of autophagy paracetamol stimulated by Lactobacillus activate the cytotoxicity of HepG2 cells [23].

Lactobacillus species targeted the anti-proliferation of colon cancer cells [24]. *Lactobacillus fermentum* has certain antioxidant, anti-proliferative and apoptosis properties , and has been revlead to have certain properties to reduce the risk of cancer [3].

Program cell death can be activated by internal or external pathways.

Our finding indicate that the cell-free supernatant of *Lactobacillus acidophillus* may induce apoptotic cell death. [26,27].

L. acidophilus produce active molecules that inhibit tumor initiation in colon cancer and of tumor in animals models. Additionally.Differences in Lactobacillus potential activity recorded by many studies indicate that lactic acid bacteria activity may be affected by different factors, such as production conditions(fermentation conditions, technology ,concentration), production conditions (strain, product active compounds)[28].

Another explantation of lactic acid bacteria effect on colon cancer may be results to production exoplysaccharides that most grame positive bacteria have. In addition to the , surface carbohydrate polymers with diverse biological functions. L.acidophillus showed ant cancer properties may be results of induction apoptosis pathway as we record in this study or cell cycle arrest in addition to the anti-oxidative, anti-angiogenesis and anti-inflammatory effects. [29].

References

[1] Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer. 2013;49:1374–1403. doi: 10.1016/j.ejca.2012.12.027. [PubMed] [CrossRef] [Google Scholar]

[2] Hanahan D, and Weinberg R. Hallmarks of cancer: the next generation. Cell. 2011;144:646–646. http://www.cell.com/abstract/S0092-8674%2811%2900127-9. [PubMed]

[3] Raguz S, Yagüe E. Resistance to chemotherapy: new treatments and novel insights into an old problem. Br J Cancer. 2008;99:387–391. doi: 10.1038/sj.bjc.6604510. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

[4] Paulo E, Boffo E, Branco E, Valente E, Melo E, Ferreira A, Roque M, Assis S. Production, extraction and characterization of exopolysaccharides produced by the native Leuconostoc pseudomesenteroides R2 strain. An Acad Bras Cienc. 2012;84:495–508. doi: 10.1590/S0001-37652012000200018. [PubMed] [CrossRef] [Google Scholar]

[5] Patel S, Majumder A, Goyal A. Potentials of exopolysaccharides from lactic acid bacteria. Indian J Microbiol. 2012;52(1):3–12. doi: 10.1007/s12088-011-0148-8. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

[6] Joo Seo B, Bajpai V, Rather I, Park Y. Partially purified exopolysaccharide from *Lactobacillus plantarum* YML009 with total phenolic content, antioxidant and free radical scavenging efficacy. Ind J Pharm Educ Res. 2015;49(4):282–292. doi: 10.5530/ijper.49.4.6. [CrossRef] [Google Scholar]

[7] Wang C, Wu T, Hsieh S, Tsai Y, Yeh C, Huang C. Antioxidant activity and growth inhibition of human colon cancer cells by crude and purified fucoidan preparations extracted from Sargassum cristaefolium. J Food Drug Anal. 2015;23:766–777. doi: 10.1016/j.jfda.2015.07.002. [PubMed] [CrossRef] [Google Scholar]

[8] Lohr H, Goergen B, Buschenfelde K, Gerken G. HCV replication in mononuclear cells stimulates anti-HCV-secreting B cells and reflects nonresponsiveness to interferon-alpha. Med Virol. 1995;46:314–320. doi: 10.1002/jmv.1890460405. [PubMed] [CrossRef] [Google Scholar]

[9] Sheng Y, Yang L, Xiaodong H, Guangxin Y, Ming G. Purification, chemical characterization and in vitro antioxidant activities of alkali-extracted polysaccharide fractions isolated from the fruits of *Schisandra chinensis*. J Med Plant Res. 2011;5:5881–5888. [Google Scholar].

[10] Gonzalez-Gil G, Thomas L, Emwas A-H, Lens PNL, Saikaly PE. NMR and MALDI-TOF MS based characterization of exopolysaccharides in anaerobic microbial aggregates from full-scale reactors. Sci Rep. 2015;5:14316. doi: 10.1038/srep14316. [PMC free article] [PubMed] [CrossRef] [Google Scholar].

[11] El-Aasr M, Kabbash A, El-Seoud K, Al-Madboly L, Ikeda T. Antimicrobial and immunomodulatory activities of flavonol glycosides isolated from *Atriplex halimus* L. Herb J Pharm Sci Res. 2016;8:1159–1168. [Google Scholar].

[12] Borenfreund E, Puerner J. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett. 1985;24:119–124. doi: 10.1016/0378-4274(85)90046-3. [PubMed] [CrossRef] [Google Scholar]\

[13] Koch A, Tamez P, Pezzuto J, Soejarto D. Evaluation of plants used for antimalarial treatment by the Maasai of Kenya. J Ethnopharmacol. 2005;3:95–99. doi: 10.1016/j.jep.2005.03.011. [PubMed] [CrossRef] [Google Scholar].

[14] Kasibhatla S, Amarante-Mendes GP, Finucane D, Brunner T, Bossy-Wetzel E, Green DR. Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis. CSH Protoc. 2006;3:4493. [PubMed] [Google Scholar].

[15] Léonce S, Pérez V, Lambel S, Peyroulan D, Tillequin F, Michel S, Koch M, Pfeiffer B, Atassi G, Hickman JA, Pierré A. Induction of cyclin E and inhibition of DNA synthesis by the novel acronycine derivative S23906-1 precede the irreversible arrest of tumor cells in S phase leading to apoptosis. Mol Pharmacol. 2001;60:1383–1391. doi: 10.1124/mol.60.6.1383. [PubMed] [CrossRef] [Google Scholar].

[16] Sawada G, Raub J, Decker D, Buxser S. Analytical and numerical techniques for the evaluation of free radical damage in cultured cells using scanning laser microscopy. Cytometry. 1998;25:254–262. doi: 10.1002/(SICI)1097-0320(19961101)25:3<254::AID-CYTO6>3.0.CO;2-F. [PubMed] [CrossRef] [Google Scholar].

[17] El-Adawi HI, Khalil MA, El-Sheekh MM, EL-Deeb NM, Hussein MZ. Cytotoxicity assay and antioxidant activities of the lactic acid bacterial strains. Afr J Microb Res. 2012;6(8):1700–12. http://www.academicjournals.org/journal/AJMR/article-abstract/ABBF3E514123.

[18] Jansson P, Stenutz R, Widmalm G. Sequence determination of oligosaccharides and regular polysaccharides using NMR spectroscopy and a novel web-based version of the computer program CASPER. Carbohydr Res. 2006;341:1003–1010. doi: 10.1016/j.carres.2006.02.034. [PubMed] [CrossRef] [Google Scholar].

[19] Lundborg M, Widmalm G. Structural analysis of glycans by NMR chemical shift prediction. Anal Chem. 2011;83:1514–1517. doi: 10.1021/ac1032534. [PubMed] [CrossRef] [Google Scholar].

[20] Rönnols J, Pendrill R, Fontana C, Hamark C, d'Ortoli T A, Engström O, Ståhle J, Zaccheus MV, et al. Complete 1H and 13C NMR chemical shift assignments of mono- to tetrasaccharides as basis for NMR chemical shift predictions of oligosaccharides using the computer program CASPER. Carbohydr Res. 2013;380:156–166. http://www.sciencedirect.com/science/article/pii/S0008621513002590. [PubMed].

[21] Dutton G, Merrifield E. The capsular polysaccharide from Kiebsiella serotype K54; location of the 0-acyl groups, and a revised structure. Carbohydr Res. 1982;105:189–203. doi: 10.1016/S0008-6215(00)84967-8. [PubMed] [CrossRef] [Google Scholar].

[22] Cescutti P, Cuzzi B, Herasimenka Y, Rizzo R. Structure of a novel exopolysaccharide produced by *Burkholderia vietnamiensis*, a cystic fibrosis opportunistic pathogen. Carbohydr Polym. 2013;94:253–260. doi: 10.1016/j.carbpol.2013.01.047. [PubMed] [CrossRef] [Google Scholar].

[23] Wang F, Yang H, Wang Y. Structure characterization of a fucose-containing exopolysaccharide produced by *Enterobacter cloacae* Z0206. Carbohydr Polym 2013;92:503–509. http://www.sciencedirect.com/science/article/pii/S0144861712010326. [PubMed].

[24] Perry B, MacLean L, Patrauchanb M, Vinogradova E. The structure of the exocellular polysaccharide produced by *Rhodococcus* sp. Carbohydr Res. 2007;342:2223–2229. http://www.sciencedirect.com/science/article/pii/S0008621507003242. [PubMed].

[25] Fotakis G, Timbrell A. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett. 2006;5:171–177. doi: 10.1016/j.toxlet.2005.07.001. [PubMed] [CrossRef] [Google Scholar].

[26] Fautz R, Husein B, Efstathiou E, Hechenberger-Freudl C. Assessment of the relation between the initial viability and the attachment of freshly isolated rat hepatocytes used for the in vivo/in vitro DNA repair assay (UDS) Mutat Res. 1993;291:21–27. doi: 10.1016/0165-1161(93)90013-P. [PubMed] [CrossRef] [Google Scholar].

[27] Nami Y, Haghshenas B, Haghshenas M, Abdullah N, Khosroushahi A. The prophylactic effect of probiotic Enterococcus lactis IW5 against different human cancer cells front. Microbiol. 2015;6:1317. [PMC free article] [PubMed] [Google Scholar].

[28] Hong, E. H. *et al*. Intratumoral injection of attenuated Salmonella vaccine can induce tumor microenvironmental shift from immune suppressive to immunogenic. *Vaccine*. **31**(10), 1377–84, https://doi.org/10.1016/j.vaccine.2013.01.006 (2013).

[29] J Wu, Y Zhang, L Ye, C Wang .The anti-cancer effects and mechanisms of lactic acid bacteria exopolysaccharides in vitro: A review.- Carbohydrate Polymers,Vol.253, 2021. https://doi.org/10.1016/j.carbpol.2020.117308.