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Evaluation of Antioxidant and Antitumor Activities of *Lactobacillus acidophilus* Bacteria Isolated from Egyptian Infants

[1]Khalid Abd EL Ghany, [1]Ekbal Abd Elhafez, [2]Ragaa A. Hamouda, [2]Hoda Mahrous,
[1]Fattah Allah H. Ahmed and [2]Hanafy A. Hamza
[1]National Organization of Drug Control and Research (NODCAR), Egypt
[2]Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt

Abstract: Probiotic bacteria are considered beneficial microorganisms and have been widely used. They protect against harmful bacteria that can cause diseases. This study was carried out to evaluate the potential for *L. acidophilus* intact cells and cell lysates as antioxidant activities and also to evaluate the potential of this bacteria and their exopolysacchariedes (EPS) as antitumor activity *in vitro* and *in vivo* against Ehrlich Ascites Carcinoma (EAC) cell line. *Lactobacillus acidophilus* demonstrated having antioxidant activities for both cell lysates and intact cells with increasing antioxidative properties for cell lysates against 2, 2-diphenyl-1-picrylhydrazyl (DPPH), also resistance to hydrogen peroxide and hydroxyl radical. The EPS produced by *L. acidophilus* showed a powerful antitumor effect *in vivo* and *in vitro* in comparison with *L. acidophilus* itself while, *E. coli* increasing solid tumor volume than control.

Key words: Antioxidant, antitumor, *Lactobacillus acidophilus*, *Escherichia coli*, exopolysccharide

INTRODUCTION

Cancer is a group of diseases characterized as uncontrolled cellular proliferation and differentiation (Ponder, 2001). Cancer is one of the most prevalent groups of disorders in populations of many countries worldwide (Jamal *et al*., 2006). Most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells and cause immunotoxicity which affects not only tumor development but also aggravate patient's recovery (Zandi *et al*., 2010). The discovery and identification of new antitumor drugs with low side effects on immune system has become an essential side effect in many studies of immunopharmacology (Xu *et al*., 2009). With this aim, much attentions had been paid to natural compounds in plants, marine organism and microorganisms. Regarding the low side effects of plants and other natural compounds, scientists are interested in working on them to find new medications (Zandi *et al*., 2010). Lactic Acid Bacteria (LAB) is considered beneficial microorganisms and have been widely used. The potential benefits of lactic acid bacteria for human health include improvement on lactose intolerance, prevention of intestinal infection, reduction of serum cholesterol, stimulation of the immune system, anticarcinogenic action and antioxidative effects (Fernandes and Shahani, 1989; Gilliland, 1990; Lin, 1995; Lin and Yen, 1999; Hitchins and McDonough, 1989; Sanders, 1993). Studies have suggested that utilization of *Lactobacilli* in foodstuffs and medicines prevents infection by pathogenic bacteria (Reid and Burton, 2002; Chen *et al*., 2005) as well as cancer formation (Bolognani *et al*., 2001). The precise mechanisms by which these organisms exert the anti-tumorigenic effects are uncertain. Probiotics may retard colon carcinogenesis by influencing metabolic, immunological and protective functions within the colon and it is possible that they may stimulate tumor cell apoptosis. (Butler *et al*., 1999). The antioxidative effect of lactic acid bacteria has been reported only recently (Lin and Yen, 1999; Ahotupa *et al*., 1996; Sanders *et al*., 1995). Many reports suggested that LAB and their fermented products have anti-tumor effects (Schiffrin *et al*., 1995). The fermented products of some LABs, such as Bifidobacterium infant's and *L. acidophilus* are known to have anti proliferative effect against the growth of breast cancer cells (Schiffrin *et al*., 1995). Kefir (YK-1) has the ability to activate the immunosuppressive response from spleen cells of a mouse when treated with immuno- suppressived substances against Ehrlich carcinoma (El-Gawad *et al*., 2004). In addition, certain cancer preventing LABs strains, such as *L. casei* and *L. acidophilus* expolysacharides (EPSs) that had received a lot of attention on their contribution to improvement in texture and viscosity of fermented food products. Also, the lactic acid bacteria produce EPS probably as a protective function of their natural environment such as against desiccation,

Corresponding Author: Khalid Abd EL Ghany, National Organization of Drug Control and Research (NODCAR), Egypt

phagocytosis, phage attack, osmotic stress, antibiotics or toxic compounds (Ruas-Madiedo *et al*., 2002). The EPS may have a role in cell recognition, adhesion to surfaces and formation of biofilms that facilitate colonization to various ecosystems, this is a beneficial attribute for probiotics in their endeavor to colonize the gastrointestinal tract. Health benefits have been attributed to some exopolysaccharides. They have been reported to possess antitumor, anti-ulcer, immune modulating and cholesterol lowering effects and anti oxidative stress of *L. acidophilus* (De Vuyst and Degeest, 1999).

So the aim at the present study is to investigate the antitumor and antioxidant activity of *L. acidophilus* bacteria and its exopolysccharides as natural agents against EAC cell line *in vitro* and *in vivo*.

MATERIALS AND METHODS

Microbial strains: Probiotic strain candidate (one strain of *L. acidophilus* P185) was identified by Mahrous (2006) which was isolated from healthy, breast- feeding infants (15 day old) and used after selection and identification according to Bergey's Manual of Determinative Bacteriology, 9th edition (Holt *et al*., 1994) with confirm identification by SDS-PAGE technique and API System. The strain was tested for its probiotic characteristics i.e., gastric acid resistance, bile salt tolerance, antibacterial activity, adhesion to human mucus. *Lactobacillus* strain was cultivated in MRS (de Man Rogosa Sharpe) broth (Lab M, IDG, UK) and incubated at 37°C in BBL anaerobic jar (Becton Dickinson Microbiology Systems, Sparks, MD) provided with disposable BBL gas generating pack (CO_2 system envelopes, Oxoid, Ltd., West Heidelberg, Victoria, Canada).

The culture was preserved in reconstituted skim milk in eppindorf tubes, stored at -80°C with glycerol (20%, v/v). Prior to use, strain was a subcultured (1%, v/v) twice in MRS broth and adjusted at 1×10^9 CFU mL^{-1}.

The *E. coli* (10536) strain obtained from ATCC was cultivated in nutrient broth (Lab M, IDG, UK) and incubated at 37°C for 24 h and preserved in reconstituted skim milk in eppindorf tubes.

Extraction of exopolysccharides from *L. acidophilus*: The EPS was isolated and purified according to Cerning *et al*. (1994) with some modification. The growth culture was heated at 100°C for 5 min to inactivate enzymes potentially capable of polymer degradation and the cells were removed by centrifugation at 8000 rpm for 5 min at 4°C. The EPS was precipitated using 2 vols of absolute ethanol. After standing overnights at 4°C, the resultant precipitate were collected by centrifugation at 8000 rpms for 20 min. The EPS was dissolved in deionized water, dialyzed against deionized water at 4°C for 24 h and freeze-dried. The freeze-dried powder was dissolved in 10% (w/v) trichloroacetic acid to remove proteins. The supernatant was dialyzed at 4°C against deionized water for 5 days and freeze-dried. These preparations were referred to as purified EPS and were stored at 4°C.

HPLC analysis: The EPS sample was analysed using a High Performance Liquid Chromatograph (HPLC) under the following conditions; mobile phase: 0.65 m msulfuric acid; columns: Aminex HPX-87H (30χ7.8 mm); temperature 75°C, flow rate, 0.7 mL min^{-1}, injection volume: 20 μL, detection at UV 205 nm by using refrence standard from sigma-addrich compony, D-(+) glucose (G8270), D-(+)glactose (G5388) and D-glucoronic acid (G5269).

Cell lysates and intact cells: Cells were harvested by centrifugation at 4°C for 30 min (5,000 g) after overnight incubation at 37°C and the pellet was washed twice with 20 mM sodium phosphate buffer (SPB, pH 7.4), then re-suspended in SPB. Washed cell suspension was disrupted with a ultrasonic cell disrupter (Brandson 4°C) and filtration (0.45 μm, Millipore). Cell debris was removed from centrifugation (10,000 g for 10 min and concentration was measured by the Bradford method (Bio-Rad Laboratories) and adjusted to 1 mg mL^{-1}. For the preparation of intact cells, cells were washed twice with SPB and re-suspended in SPB. The total cell number was adjusted to 10^9 CFU mL^{-1} (Kim *et al*., 2006).

Resistance to hydrogen peroxides and hydroxyl radical: Hydrogen peroxides and hydroxyl radical were observed by the method of Kullisaar *et al*. (2002). For the measurement of *Lactobacilli* resistance in the presence of hydrogen peroxide, cells were suspend at the level of 10^7 CFU mL^{-1} in Sodium Phosphate Buffer (SPB) and incubated with 1.0 mM hydrogen peroxide (30%) at 37°C. At 1 h time intervals, the number of viable cells was estimated at MRS agar plates. For resistance to hydroxyl radicals, cells (10^7 CFU mL^{-1}) were incubated with the solution containing 10 mM THAs (terephthalic acid, Sigma) in SPB and 0.01 mM $CuSO_4\times5H_2O$. The reaction was started by the addition of 1 mM hydrogen peroxide and the number of viable cells was estimated (Kim *et al*., 2006).

For blanks, the cells were suspended at the level of 10^7 CFU mL^{-1} in SPB and incubated at 37°C for 7 h. At 1 h time intervals, the number of viable cells was estimated on MRS agar plates and we examined that the number of viable cell did not affect for 7 h.

DPPH freed radicals scavenging assay: Evaluation of Antioxidant activity by *in vitro* technique with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. DPPH (0.1 Mm) was prepared by dissolving 1.9 mg from DPPH to absolute methanol in 100 mL, then keeping in the dark place for 1 h and 1.0 mL of this solution was added to 3.0 mL of extract solution in methanol at different concentrations. Thirty minute later, the absorbance was measured at 517 nm. A blank was prepared for adding extract. Concentrations (1-18 μg mL^{-1}) were used as standard; lower absorbance of the reaction mixture indicates higher free radicals scavenging activity. The scavenging ability was defined as follows:

$$1-\frac{A517\ (sample)}{A517\ (blank)}\times 100$$

***In vitro* assessment of anti tumor activity of *L. acidophillus* bacteria and its EPS**

Tumor cells: The initial inoculums of Ehrlich Ascites Carcinoma (EAC) were purchased from the National Cancer Institute, Cairo University, Egypt. EAC cells were propagated in NODCAR laboratories by weekly intraperitoneal injection of 0.2 of 1:5 mL^{-1} saline solution of freshly drawn ascetic fluid (0.2×10^6 EAC cells) from a donor mouse bearing 6-8 day old ascitic tumor, into three mice to ensure that the ascetic fluid will still propagated and can then be drawn from atleast on life mouse. Transplantation was carried out using sterile disposable syringes under aseptic conditions. The tumor growth was as rapid as to killing the mice within 18-20 day due to the accumulation of ascetic fluid and rarely the tumor showed distal metastasis or spontaneous regression. After making appropriates dilution, the non-viability was tested for trypan blue exclusion method, (Freshney, 2005), as shown follows:

$$\frac{\text{No. of non-viable cells}}{\text{Total cells}}\times 100$$

By this method we can determine the effective dose of bacterial extracts induced more regression of tumors.

Effect of different doses from bacteria on the growth of solid tumors in male albino mice: Adult Male Swiss albino mice with an average body weight 20-25 g were used as experimental animals throughout the study. The mice were housed in specially designed cages and maintained in a thermostatically controlled room during the experimental period. They were fed an ordinary pellet diet and given up tap water ad-libitum in (NODCAR) these mice were divided into 4 groups having 5 animals each:

Group 1: Healthy mice injected subcutaneous in thigh with 1×10^6 tumors cell/mouse (control)

Group 2: Healthy mice oral inoculated with daily dose of 500 μg mL^{-1} from *L. acidophilus* (1×10^9) CFU for 14 day, then the animals injected with 1×10^6 tumor cell/mouse subcutaneous, then oral inoculated by *L. acidophilus* (1×10^9) CFU three dose weekly for two week

Group 3: Healthy mice injected intra peritoneal with daily dose of 500 μg mL^{-1} of conc. 1 mg mL^{-1} from EPS extracted from *L. acidophillus* then the animals injected with 1×10^6 tumor cell/mouse subcutaneous and injected with EPS three times weekly for two week

Group 4: Healthy mice oral inoculated with daily dose 250 μg mL^{-1} *Escherichia coli* (1×10^6) CFU mL^{-1} for 14 day, then some animals injected with 1×10^6 tumor cell subcutaneous, then oral inoculated by three dose *E. coli* (1×10^6) CFU mL^{-1} weekly for two week

Statistical analysis: Data obtained was subjected to analysis of variance and the means were compared using the Least Significant Differences (LSD) tested for the 0.05 level, as recommended by Snedcor and Cochran (1982). *In vitro* experiments were conducted in triplicate.

RESULTS AND DISCUSSION

Resistance of *L. acidophilus* bacteria to hydroxyl radicals: Figure 1 and 2 show the resistance of *L. acidophillus* to hydrogen peroxide and hydroxyl radicals. *Lactobacillus acidophilus* was viable for 7 h in concentrations of 1.0 mM h ydrogen peroxides and 0.01 mM hydroxyl in the presence of highly damaging effect, both these intact cell and cell lysate, had strong anti-oxidatived activities with increasing inhibition percentage in cell lysate than intact cell. Anti-oxidatived activity of microorganisms is one of the reasons for their increased resistance to Reactive Oxygen Species (ROS). Kullisaar *et al.* (2002) reported that the antioxidant strain *L. acidophilus* had significantly high resistance to ROS. Kim *et al.* (2006) proved that *L. acidophilus* was the most active strain which hydroxyl radicals scavenging activity in cell lysates was 70 and 53% in intact cells. The scavenging of different types of ROS was thought to be one of the main antioxidant mechanisms of the antioxdant action exhibited by lactic acid bacteria (Namiki, 1990).

DPPH method: DPPH is a relatively stable organic radical, it has been widely used in the determination of antioxidant

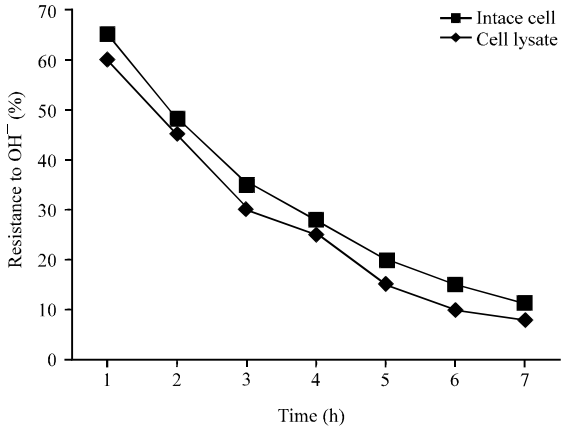

Fig. 1: Perecantage of resistance of *L. acidophilus* bacteria to hydrogen peroxide. Bacteria was suspended at the level of 10^7 CFU mL^{-1} with conc. 1 mg mL^{-1} for both cell lysates and intact cells and incubated with 1.0 mM hydrogen peroxides

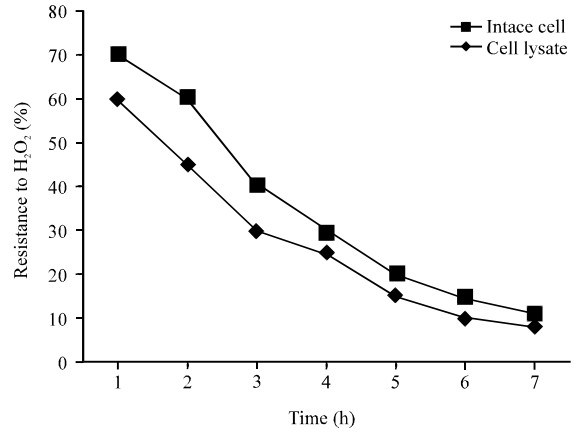

Fig. 2: Perecantage of resistance of *L. acidophilus* to hydroxyl radical. Bacteria was suspended at the level of 10^7 CFU mL^{-1} with conc. 1 mg mL^{-1} for both cell lysates and intact cells and incubated with 0.01 mM hydroxyl radicals

activities. Figure 3 shows the results of DPPH scavenging potentioal of different concentrations of *L. acidophillis*, cell lysates and intact cells. The maximum antioxidant activity was 69.41 and 65.215% with 80 μg mL^{-1} of both cell lysates and intact cells. It is noticed that the antioxidant activaty of different concentrations of cell lysates was more effective than different concentrations of intact cells. Lin and Chang (2000) indicated that the radical scavenging ability of the intact cells and intracellular extracts of *B. longum* and *L. acidophilus* contribute good antioxidative effect on inhibiting linoleic acid peroxidation and scavenging the DPPH radical.

Chemical composition of exopolysaccharides (EPS): The results of exopolysaccharides content secreted by *L. acidophilus* are shown in Fig. 4, which shows different sugar content and concentrations. The sugar contents is glucose, galactose and D. glucoronic acid (71.9, 22.46 and 0.16 mg, respectively). The chemical composition of exopolysaccharides from *L. acidophilus* has been investigated and there is agreement that EPSs from LABs is polysaccharides with D. glucose and

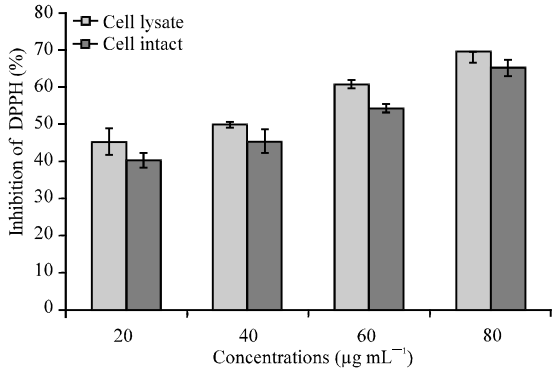

Fig. 3: Antioxidant activities of different concentrations of *L. acidophillis* cell lysates and intact cells against DPPH radical

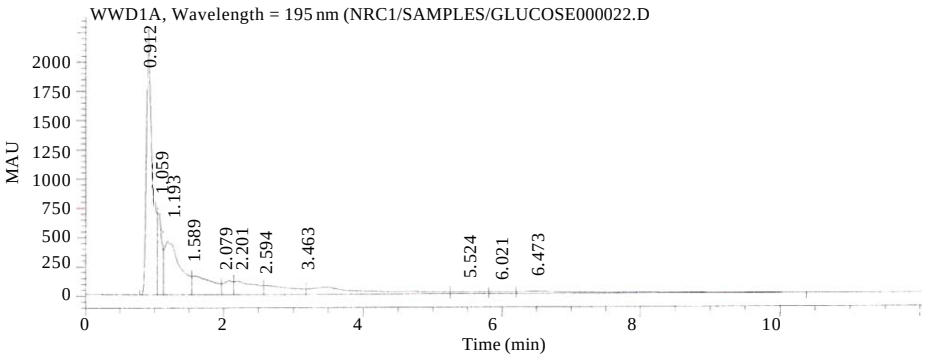

Fig. 4: EPS by HPLC analyses containing D. glucoronic acid, D. glactose and D. glucose

Table 1: *In vitro* cytotoxic effect of different concentrations of *L. acidophilus* and their exopolysaccharides on the non viability of Ehrlich Ascites Carcinoma (EAC) cells

EPS (%)	*L. acidophillus* non-viable (EAC)%	Concentration (μg mL^{-1})
5	4	100
25	20	200
40	35	300
71	60	400
89	69	500
94	87	600
99	93	700

Table 2: Effect of tested *Lactobacilli* and its exopolysaccharides on the volume of solid tumor (mm^3)

Days	G1 control	G2 L. A	G3 EPS	G4 *E. coli*
10	0.30±0.010	0.21±0.033	0.21±0.035	0.25±0.040
11	0.32±0.009	0.30±0.021	0.30±0.018	0.33±0.033
12	0.38±0.020	0.38±0.021	0.39±0.025	0.42±0.023
13	0.47±0.028	0.47±0.027	0.47±0.028	0.51±0.009
14	0.53±0.034	0.52±0.031	0.53±0.026	0.61±0.011
15	0.59±0.040	0.59±0.025	0.60±0.027	0.68±0.011
16	0.69±0.029	0.67±0.027	0.68±0.030	0.79±0.027
17	0.75±0.066	0.73±0.014	0.74±0.018	0.91±0.036
18	0.87±0.103	0.77±0.011	0.78±0.011	1.11±0.097
19	1.09±0.151	0.80±.025	0.83±0.012	1.45±0.139
20	1.75±0	1.02±0.022***	1.21±0.019***	2.05±0***

***Highly significant with p≤0.05

D. galactose as main sugar constituents and the ratio of the two components varies (Laws *et al*., 2001).

***In vitro* assessment of the effective dose of *L. acidophilus* and its EPS against EAC cells:** Different concentrations of *L. acidophilus* and its EPS (100, 200, 300, 400, 500, 600 and 700 μg mL^{-1}) was investigated on the non viability of Ehrlich Ascites Carcinoma (EAC) cell line as shown in Table 1. Results showed that different concentrations of *L. acidophilus* and its EPS inhibited the proliferation of EAC cells. The EPS showed more antiproliferatives effects against EAC cell line than *L. acidophilus*. The maximal precentage of inhabition of EAC cells line was (93 and 99%) with 700 μg mL^{-1} of *L. acidophilus* and its EPS, respectively. This result is demonstrated by Doleyres and Lacroix (2005) which reported LAB EPS is provided beneficial physiological effects on human health, such as antitumours activity, immunomodulating bioactivity and antimutagenicity.

Effect of *L. acidophilus* and its exopolysaccharides on the volume of solid tumors: Further *in vivo* study for the highly toxic *L. acidophilus* and its exopolysaccharides has been done on solid tumor bearing mice. As show in Fig. 5 and Table 2, *L. acidophilus* and its exopolysaccharides caused significant reduction in the tumor volume as compared to that of the positive control group and effects of *E. coli*. The reduction of tumor volume (1.02±0.022 and 1.21±0.019 mm^3) were observed when mice-bearing solid tumor was treated with exopolysaccharides extracted from *L. acidophilus*. The EPS from *L. acidophilus* showed powerful

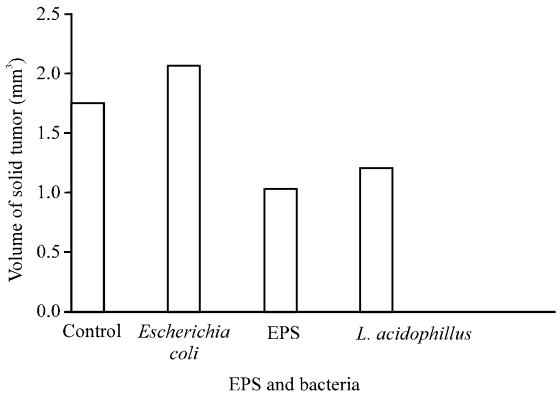

Fig. 5: Effect of tested *Lactobacilli* and its exopolysaccharides on the volume of solid tumor (mm^3)

reduction of solid tumor volume than *L. acidophilus* itself while *E. coli* causing propagation of solid tumor volume than control mice. Baldwin *et al*. (2010) suggested that probiotics may be used as adjuvants in anticancer chemotherapy. Daniluk (2012) reported that the anticancer activity through induction into a poptosis of cancer cells seems to be promising to approach for use of some probiotic strains on support therapy or disease prevention. Probiotics also have a role in prevention of colon cancer (Dugas *et al*., 1999; Rafter, 2003). Different strains of probiotics had different effects on the intestinal luminal environment, epithelial and mucosal barrier function and the mucosal immune system (Hirayama and Rafter, 2000).

CONCLUSION

This study demonstrated that *L. acidophilus* having antioxidant activities for both intact cells and cell lysates. The *L. acidophilus* and its exopolysccharide (EPS) had antitumor activaty *in vivo* and *in vitro*. The exopolysccharide of *L. acidophilus* is more effective against Ehrlich Ascites Carcinoma (EAC) cells than *L. acidophillus*.

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