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Detection on Antioxidant and Cytotoxicity Activities of Exopolysaccharides Isolated in Plant-Originated *Lactococcus lactis*

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ABSTRACT

The aim of the present study was to determine whether water - soluble polysaccharides (EPS) of *Lactococcus lactis* NCR112 isolated in *Phyllanthus urinaria* could express the inhibition of human cancer cells as well as the antioxidant activity. The water - soluble polysaccharide of *L. lactis* NCR112 was studied on the antioxidant activity by using DPPH radical scavenging assay and sulforhodamine B (SRB) assay for anticancer activity tests on two HeLa and Hep G2 tumor cell lines. The highest crude EPS amount was obtained at the stationary phase. The crude EPS (10 mg/ml) showed the antioxidant activities with the inhibition percentage of 66.10 ± 1.157, equaled to the activity of ascorbic acid at 0.01315 mg/ml. The crude EPS of *L. lactis* NCR112 also showed the higher cytotoxicity percentage on HeLa (86.86 ± 4.875) than Hep G2 (50.36 ± 6.237). These results indicated that the exopolysaccharide isolated from *L. lactis* NCR112 constituted the major fraction that inhibited the proliferation of cancer cells. This was the first report on antioxidant activity of plant – originated *L. lactis* NCR112.

Key words: Antioxidant activity, anticancer activity, DPPH radical scavenging assay, *L. lactis* NCR112, sulforhodamine B (SRB).

INTRODUCTION

Most of diseases are due to the "oxidative stress" resulting from an imbalance between formation and neutralization of pro-oxidants. Oxidant stress is initiated by free radicals, which produced aerobic metabolism in the body, can cause oxidative damage of biological macromolecules such as proteins, lipids, and DNA in healthy human cells (Yen and Chen, 1995; Gutteridge and Halliwell, 1993; Halliwell, 1995). These changes contribute to oxidative stress that is among the major causative factors in the induction of many chronic and degenerative diseases including atheorosclerosis, ischemic heart diseases and diabetes mellitus, cancer, immunosuppression, neurodegenerative disease, ageing (Squadrito and Pryor, 1998; Devasagayam *et al.*, 2004; Büyükokurolu *et al.*, 2001; Shahidi *et al.*, 1992; Gülçin *et al.*, 2002; Branen, 1975), coronary heart disease and Alzheimer's disease (Ames, 1983; Gey, 1990; Smith *et al.*,1996; Diaz *et al.*, 1997). All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase and catalase, or compounds such as ascorbic acid, toccopherol and glutathione (Niki, 1994). However, these protective mechanisms occurred by various pathological processes, and antioxidant supplements are necessary to combat oxidative stress. Currently, the well-known synthetic antioxidants like butylated hydroxylanisole (BHA) and butylated hydroxytoluence (BHT), tertiary butulated hydroquinon and galic acid esters, are reported to cause or promote releasing carcinogens. Therefore, the interest in the natural compounds with strong antioxidant properties has steadily been increasing. Furthermore, the plant-originated lactic acid bacteria such as *L. rhamnosus* PN04 showed the strong biological activities (Nguyen *et al.*, 2013;

2014). Meanwhile, *L. lactis* strains isolated in milk and food have several beneficial effects, such as antimicrobial activity (Khalid *et al*., 2011; Elliott *et al*., 1996; Roy *et al*., 1996; Rodr1guez *et al*., 2005), ability to modulate immune response (Fang *et al*., 2000; Chatel *et al*., 2011), anti-tumorigenic activity (Shalke, 2013; Mohammadi, 2013) and antioxidant activity (Virtanen *et al*., 2007). It has been shown that *L. lactis* possessed antioxidant activity that was able to decrease the risk of accumulation of reactive oxygen species during ingestion of food (Pan and Mei, 2010; Rochat *et al*., 2005).

As the above stated reasons, study on the antioxidant and anticancer activities of plant – originated *Lactococcus lactis* NCR112 was necessary because this strain adapted the harsh condition than the animal originated *L. lactis.*

MATERIALS AND METHODS

Culture *Lactococcus lactis* NCR112 at difference growth phase

Lactobacillus lactis NCR112 isolated from *Phyllanthus urinaria* was deposited in DDBJ under the accession number (AB828399). This strain was cultured in De Man-Rogosa- Sharpe (MRS) (Biokar Diagnostics, Beauvais, India) and incubated at 37°C under aerobic conditions (pH 6.5). The optical density (OD) measurement at wavelength of 600 nm was performed every two hours.

Preparation of polysaccharides

Lactococcus lactis NCR112 was cultured in De Man-Rogosa- Sharpe (MRS) (Biokar Diagnostics, Beauvais, India) and incubated at 37°C under aerobic conditions. Cultures were collected at different phase of incubation and centrifuged at 10000 rpm for 30 min to separate the cell from the broth. The culture supernatant was let overnight to precipitate with three times volume of absolute cold ethanol (EtOH) and then centrifuged again at 10000 rpm for 30 min. The obtained pellet was resuspended with distilled water and further precipitated by adding three times volume of cold EtOH. The overnight solution was centrifuged to collect the water-soluble exopolysaccharides (EPS). The crude EPS was dried at 60°C to a constant weight. EPS stocks were dissolved in distilled water and then filtered through the 0.22 µm pore-size filters (Millipore, Bedford, Mass.) before using.

Antioxidant activity using DPPH radical scavenging assay

In order to perform the 2,2-diphenylpi crylhydrazyl (DPPH assay), amounts of 4.3 mg of DPPH were dissolved in 3.3 ml methanol in a test tube (Padmavathy, 2014). Solution was protected from light by covering the test tubes with aluminum foil. 150 ml of above solution were added to 3ml methanol. This solution was measured at 517 nm on UV spectrophotometer. Methanol was used as blank. This reading was used as control reading. For the test and standard (ascorbic acid), the aliquots of different concentration ranging were prepared. After 50 ml of tested EPS (10 mg/ml) and standard ascorbic acid in the various concentration were diluted with methanol up to 3ml, 150 ml DPPH was added. All these samples were taken after 12 h and measured at 517 nm on UV-visible spectrometer (Shimadzu, UV-1601, Japan). The DPPH free radical scavenging activity was calculated using the following formula:

$$\frac{(1 \quad A_s)}{A_c} x\ 100$$

Where A_s and A_c are the absorbance of control and sample, respectively.

Antitumor activity of sulforhodamine B (SRB) assay

In order to perform antitumor activity test, the crude EPS sample was prepared according to the above prescribed procedure. The sulforhodamine B (SRB) assay was used in the study according to the method of Longo-Sorbello with a slight modification (Longo-Sorbello *et al*., 2005). Each cancer cell line was seeded in a 96-well plate (1.0 x 10^4 cells per well). After 24h of incubation, the test samples (115.3 x 10^6 cfu/ml of cell-free supernatant and 20 mg/ml of crude EPS) were added to the cancer cells, 5% CO_2 for 48 h at 37°C. For this incubation time, no significant differences were observed in the pH of medium. Thereafter, 50 µl of 50% TCA (4°C) was added to each well containing 200 µl of medium to reach a final concentration of 10% TCA in each well and plate the 96-well plate for 1h at 4°C to allow cell fixation. After 1 h of incubation, the culture medium in each well was removed and the plate was gently washed with water (200 µl/well)

five times and dried at room temperature for 12-24 h. 0.2% SRB (w/v) solution was added after the time for incubation to each well and leave at room temperature for 5-20 min. Then, washing the plate with 1% acetic acid was performed five times in order to remove unbound SRB. Drying the plate for 12-24 h before adding 200 µl Trizma-base 10 mM in oder to solublize bound SRB is necessary. The last step is plating the 96-well plate on a plate shaker for at least 10 min. Absorbance was measured at 492 nm and 620 nm using an enzyme-linked immunosorbent assay plate reader (Molecular Devices, Synnyvale, CA, USA). DMSO was used as a negative control. The percentage of viable cells was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{(\text{OD of control group} - \text{OD of experimental group})}{(\text{OD of control group})} \times 100$$

Statistical analyses

The SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to calculate the means and standard deviations in any experiments involving triplicate analyses of any samples. The statistical significance of any observed difference was evaluated by one-way analysis of variance (One way ANOVA), using the Bonferront Mutiple Comparisons Test.

RESULTS AND DISCUSSION

Polysaccharide collection

There have been many publications that have developed the procedure for EPS isolation. Most of these methods commonly use solvent such as acetone (Vincent *et al*., 2001; Lemoine *et al*., 1997) or ethanol (Rodriguez *et al*., 2008) to precipitate EPS. In this study, using ethanol for EPS precipitation, the crude EPS samples were collected and determined at four different phases (table 1, figure 1). Although there was less significant difference between growth phases, the weight at stationary phase gave the highest amount (0.0525 g/5ml). That might also due to the best growth correlating the EPS biosynthesis of *Lactobacillus lactis* NCR112.

Table 1: Weight of EPS at different phase of *L. lactis* NCR112

Growth phase Weight of crude	EPS (g/5ml)
Early exponential phase	0.0328 ± 0.0022[a]
Late exponential phase	0.0456 ± 0.0003[b]
Stationary phase	0.0479 ± 0.0022[b]
Death phase	0.0471 ± 0.0038[b]

Results are mean values of triplicate determinations ± SD
The sample letters in the same column are not significant different ($p < 0.05$)

Antioxidant activity tests using DPPH radical scavenging assay

In this present study, the antioxidant activity of the crude EPS of *Lactobacillus lactis* NCR112 at different phase was investigated using the DPPH scavenging assay. Scavenging activity of crude EPS produced at different phase on DPPH radical has been shown in Table 2 and Figure 2. The crude EPS (10 mg/ml) gave the antioxidant activities with the inhibition percentage of 52.86 ± 0.133 when compared with ascorbic acid activity at 13.15 µg/ml according to the standard curve ($y = 1.7204x + 43.475$, $R^2 = 0.9993$). As a result, there was noticeable antioxidant activity of the EPS fraction.

Table 2: DPPH scavenging activity of crude EPS

Growth phase	% DPPH scavenging activity of crude EPS (10 mg/ml)
Early exponential phase	42.44 ± 0.576[a]
Late exponential phase	47.71 ± 0.273[b]
Stationary phase	66.10 ± 1.157[c]
Death phase	57.62 ± 1.112[d]

Table 3: Inhibitory effects of cell-free supernatant and crude EPS at stationary phase on the growth of two human cancer cell lines

Cancer cell line	% cytotoxicity of EPS
HeLa	86.86 ± 4.875
Hep G2	50.36 ± 6.237

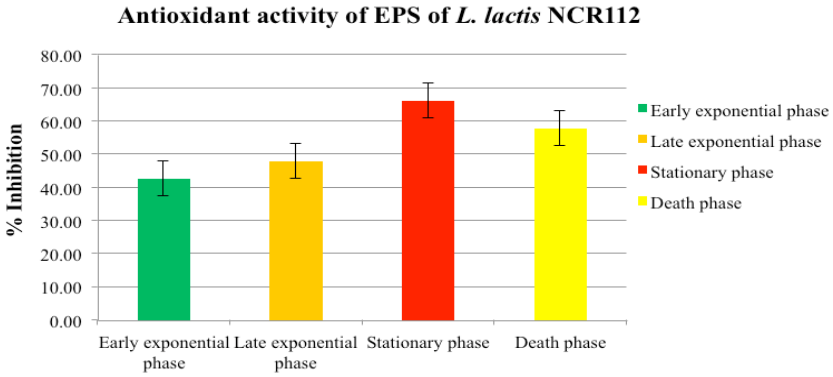

Fig. 1: Weight of crude EPS at the different growth phases

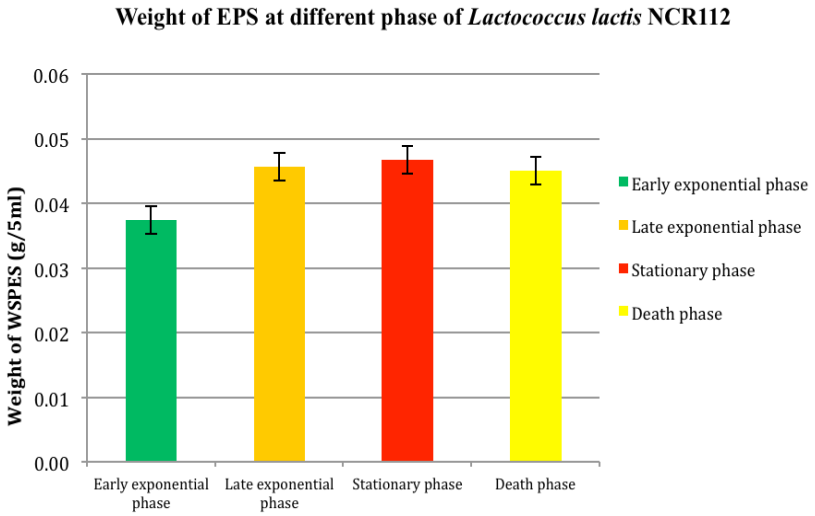

Fig. 2: The DPPH scavenging activity of crude EPS

Antitumor activity on SRB assay

From the results of antioxidant activity, it was recognized clearly that the bacterium could produce the highest amount of antioxidant activity. Consequently, the crude EPS was performed to test antitumor activity. As illustrated in table 3, crude EPS was effective to Hela and Hep G2 cancer cells. Depending on the different effect mechanism to cancer cell lines, the cytotoxicity percentage of each sample was also different. For Hela cancer cell, the cytotoxicity percentage of crude EPS were 86.86 ± 4.875. In case of Hep G2 cancer cell, the cytotoxicity percentage of crude EPS were 50.36 ± 6.237. These results indicated that the exopolysaccharide isolated from *L. lactis* NCR112 constituted the major fraction that inhibits the proliferation of cancer cells. The cytotoxicity on the different cancer cell lines that may be affected by the cancer cell receptor binding. To clearly understand this point, more studies are being performed.

CONCLUSION

The crude EPS derived from plant originated *L. lactis* NCR112 exerted significant antioxidant activity, as well as anticancer activity on two HeLa and Hep G2 cell lines. These polysaccharide components may be applied to various foods, and used as adjunction in cancer trials. In future, the optimizing conditions and components of EPS should be determined to exploit EPS for human health.

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