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Detection on Cytotoxicity Activities in *Lactobacillus rhamnosus*

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The aim of the present study was to determine whether culture supernatant and water soluble polysaccharides of *L. rhamnosus* PN04 could suppress the inhibition of human cancer cells along with the antioxidant activity. A culture supernatant and 90% ethanol extract (WSPES) of *L. rhamnosus* PN04 were studied on the antioxidant activity by using DPPH radical scavenging assay and using SRB assay for anticancer activity on two HeLa and Hep G2 human cancer cell lines. The amount of 90% ethanol extract change as the same the growth stage of *L. rhamnosus* PN04. The highest WSPES amount was obtained at the stationary phase. Both the culture supernatant ($101.5x10^6$ cfu/ml) and 10 mg/ml WSPES (at stationary phase) were the most active antioxidant with the percent inhibition of 95.19 ± 0.062 and 52.86 ± 0.133 when compared with ascorbic acid at 29.77 μg/ml and 13.15 μg/ml before respectively. So, only culture supernatant and WSPES at this phase were performed the antitumor activity test. Culture supernatant of *L. rhamnosus* PN04 were determined to be less toxic to HeLa cell lines than were Hep G2 cell lines. However, the WSPES were more effective to Hep G2 than HeLa cell lines. Therefore, both culture supernatant and WSPES of this bacteria exhibited potent antioxidative activity and anticancer activity. The present study may contribute to discover the new and natural compounds for cancer treatment.

Key words: Antioxidant activity, anticancer activity, DPPH radical scavenging assay, SRB.

Recently, more evidence suggested that many today diseases are due to the "oxidative stress" that results from an imbalance between formation and neutralization of pro-oxidants. Oxidative 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 being 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; Büyükokuroðlu *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, tocopherol and glutathione (Niki, 1994). However, these protective mechanisms are occurred by various pathological processes, and antioxidant supplements are necessary to combat oxidative stress. Currently, the well-known

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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. Besides, these agents are not only expensive, but doubts have been raised as to the long-term stability and safety. Therefore, the interest in the natural compounds with strong antioxidant properties but low cytotoxicities steadily has been increasing. Furthermore, lactic acid bacteria such as *L. rhamnosus* have several beneficial effects, such as antimicrobial activity (Pithva *et al*., 2011; Coppola *et al*., 2005; De Keersmaecker *et al*., 2006; Goyal *et al*., 2011), ability to modulate immune response (Fang *et al*., 2000; Roller *et al*., 2004; Sheih *et al*., 2001; Braat *et al*., 2004; Panigrahi *et al*., 2004; Gill *et al*., 2001; Veckman *et al*., 2004; Cross *et al*., 2002; Gill and Rutherfurd, 2001; Vizoso *et al*, 2009) and anti-tumorigenic activity (Shalke, 2013; Mohammadi, 2013) and antioxidative activity (Virtanen *et al*., 2007; Choi *et al*., 2006; Korpela *et al*., 1997; Kaizu *et al*., 1993).

MATERIALS AND METHODS

Culture *Lactobacillus rhamnosus* PN04 at difference growth phase

L. rhamnosus PN04 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 measurement at wavelength of 600 nm was performed every two hour.

Preparation of polysaccharides

L. rhamnosus PN04 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 precipated with three times of volume of 4°C absolute ethanol (EtOH). After overnight, culture supernatant was 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 4°C EtOH. The overnight solution was centrifuged again, this pellet was water-soluble exopolysaccharides (WSPES) and dried at 60°C to a constant weight. WSPES stocks were filtered through a 0.22 µm pore-size filter (Millipore, Bedford, Mass.) and stored at -80°C till used.

Antioxidant activity tests using DPPH radical scavenging assay

The scavenging ability of the methanol and aqueous samples on 1,1-diphenyl-2-picryl hydrazyl (DPPH) (Sigma-Aldrich) radicals was measured according to the method of Patel with a slight modification (Patel and Patel, 2011). The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm and purple colour existed. When antioxidant agents react with DPPH which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPH-H. Consequently, the absorbance decreased from the DPPH radical to the DPPH-H form (figure 1), resulting in the decolorization or yellow colour with respect to the number of electrons captured. The decolorization is the result of reducing ability.

The scavenging reaction between (DPPH) and an antioxidant (H-A) shown in figure 1 when 4.3 mg of DPPH was dissolved in 3.3 ml methanol. The chemical mixture was protected from light by covering the test tubes with aluminum foil. A portion of 150 ml DPPH solution was added to 3ml methanol and absorbance was taken immediately at 517nm for control reading. 50 ml of samples (10 mg/ml) as well as various concentration of standard ascorbic acid were taken and the volume was made uniformly to 150 ml using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each 150 ml DPPH was added. Absorbance was taken after 12 h at 517 nm using methanol as blank on UV-visible spectrometer (Shimadzu, UV-1601, Japan). The DPPH free radical scavenging activity was calculated using the following formula:

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

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

Sulforhodamine B (SRB) assay for antitumor activities

The Sulforhodamine B (SRB) assay is based on binding on the dye to basic amino acids

of cellular proteins, and colorimetric evaluation provides an estimate of total proteins mass, which is related to cell number. This assay has been widely used for the in vitro measurement of cellular protein content of both adherent and suspension cultures. In the present study, it was measured 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 culture supernatant and 20mg/ml of WSPES) were added to the cancer cells, 5% CO_2 for 48 h at 37°C. For this incubation time, there was no significant differences 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 fnal 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 let 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 solublize bound SRB is nescessary. 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). Dimethyl sulfoxide (DMSO) was used as a negative control. The percentage of viable cells was calculated as follows:

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

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 elauated by oneway analysis of variance (One way ANOVA), using the Bonferront Mutiple Comparisons Test.

RESULTS

Preparation of Polysaccharides

After determining the exact period growth of each phase, we chose the typical time for each phase that was shown in the table 1 and figure 2.

Antioxidant activity tests using DPPH radical scavenging assay

In this present study, the antioxidant activity of the ethanol extracts of the medium culture *L. rhamnosus* PN04 at different growth phases was investigated using the DPPH scavenging assay. Scavenging activity of culture supernatant and WSPES produced at different phase on DPPH radical has been shown in table 3, table 4 and figure 4, figure 5. Both the culture supernatant ($101.5x10^6$ cfu/ml) and 10 mg/ml WSPES (at stationary phase) showed the most active with the inhibition percentage of 95.19 ± 0.062 and 52.86 ± 0.133 when compared with ascorbic acid at 29.77 µg/ml and 13.15 µg/ml, respectively. As evidence from these results, there are noticeable variability in the antioxidant activity of samples. And the antioxidant activities exhibited by the culture supernatant was more produced than that of the WSPES fraction.

Antitumor activity using SRB assay

From the results of antioxidant activity, we recognized clearly that the stationary phase

Table 1. Weight of WSPES at difference phase

Growth phase	Weight of EPS (g/5ml)
Early exponential phase	0.0445 ± 0.0028^{a}
Late exponential phase	0.0505 ± 0.0034^{ab}
Stationary phase	0.0525 ± 0.0039^{b}
Death phase	0.0501 ± 0.0008^{ab}

Table 2. DPPH scavenging activity of ascorbic acid

		Concentration (µg/ml)					
Standard	Absorbance	5	10	15	20	25	30
Ascorbic acid	% Inhibition	52.14	60.39	64.02	78.54	86.79	94.61

Table 3. DPPH scavenging activity of supernatant

Growth phase	% DPPH scavenging activity of supernatant
Early exponential phase	92.87± 0.075[a]
Late exponential phase	94.28 ± 0.046[b]
Stationary phase	95.19 ± 0.062[c]
Death phase	94.91 ± 0.075[d]

Table 4. DPPH scavenging activity of WSPES

Growth phase	% DPPH scavenging activity of supernatant
Early exponential phase	35.68 ± 1.191[a]
Late exponential phase	36.62 ± 0.075[b]
Stationary phase	52.86 ± 0.133[c]
Death phase	51.45 ± 0.112[d]

Table 5. Inhibitory effects of culture supernatant and WSPES at stationary phase of *L. rhamnosus* PN04 on the growth of two human cancer cell lines

Cancer cell line	% cytotoxicity		
	Samples	Culture supernatant	WSPES
HeLa	-	46.99 ± 6.251	86.16 ± 4.835
Hep G2	-	64.40 ± 4.824	58.78 ± 3.677

produces the highest amount of antioxidant activity. So, only culture supernatant and WSPES at this phase were performed the antitumor activity test. As illustrated in table and figure both superantant and WSPES were effective to HeLa and Hep G2 cells. Depending on the different effect mechanism to cancer cell lines, the cytotoxicity percentage of each sample is also different. For

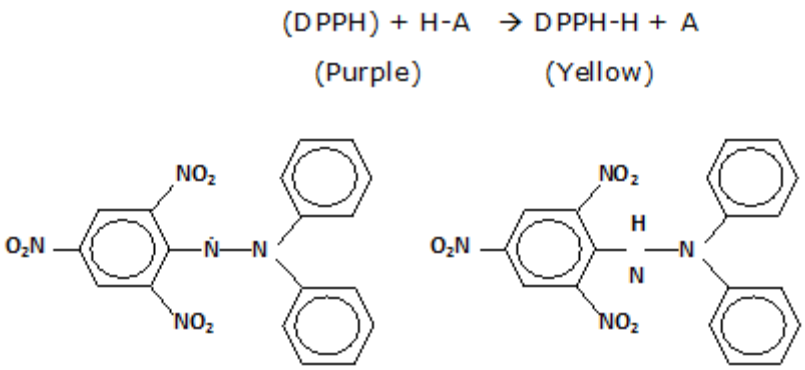

Fig. 1. Reaction of DPPH (free radical) to DPPH (Non radical)

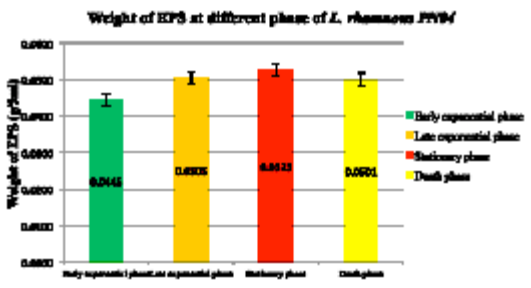

Fig. 2. Weight of WSPES at different growth phases of *Lactobacillus rhamnosus* PN04

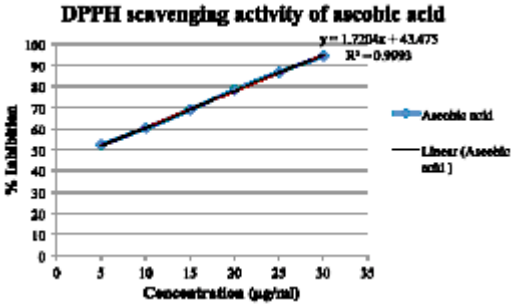

Fig. 3. DPPH scavenging activity of ascorbic acid

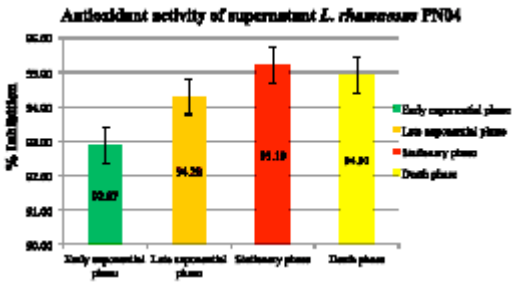

Fig. 4. DPPH scavenging activity of cell – free culture

Fig. 5. DPPH scavenging activity of WSPES

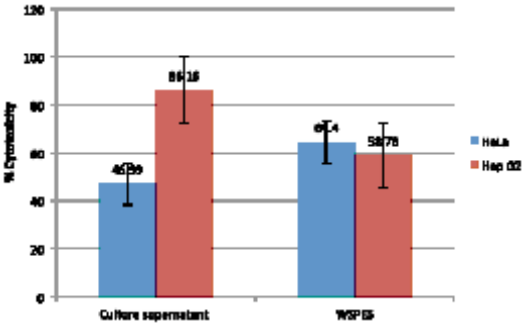

Fig. 6. Inhibitory effects of culture supernatant and WSPES at stationary phase of *L. rhamnosus* PN04 on the growth of two human cancer cell lines using SRB assay

example, culture supernatant (64.40) was more effective than WSPES (58.78) on Hep G2 cells, however, there is converse on HeLa cells with value 46.99 and 86.16 respectively. These results indicate that both culture supernatant and WSPES of *L. rhamnosus* PN04 potently inhibited the viability of each cell line.

DISCUSSION

We knew that free radicals are 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; Büyükokuroðlu *et al*., 2001; Shahidi *et al*., 1992; Gülçin *et al*., 2002; Branen, 1975), coronary heart disease, Alzheimer's disease, others (Ames, 1983; Gey, 1990; Smith *et al*.,1996; Diaz *et al*., 1997). Therefore, the results of free radical scavenging activity showed the supernatant ($115.3x10^6$ cfu/ml) and 10 mg/ml WSPES (at stationary phase) have strongest activity among other samples respectively with ascorbic acid at 29.769 µg/ml and 13.151 µg/ml (Table 4 and Figure 5). In both DPPH radical scavenging activity of supernatant and WSPES, % inhibition increases from early exponential phase to stationary phase and slightly decrease at death phase. This phenomenon indicated that a pro-oxidant effect occur (Gill and Rutherfurd, 2001). Besides, all samples gave positive scavenging capacity with DPPH. From results, *L. rhamnosus* PN04 possesses the ability of either inhibiting free radical formation or itself be a free radical scavenger. Therefore, these results of antioxidant activity may support evidence affecting of *L. rhamnosus* PN04 for cancer cell lines.

As shown in figure 6, the inhibitory effects of culture supernatant were higher than the WSPES at stationary phase of *L. rhamnosus* PN04 on two human cancer cell lines using SRB assay. It was meant that there was other cytotoxicity compounds produced in culture besides polysaccharides. More studies will be done so far to explain the phenomenon.

Currently, one of the most important factors in human longevity is the control of tumors. Therefore, studies on the beneficial effects of lactic acid bacteria have been largely focused on their antitumor effects. Most studies, however, have focused on the effects of LAB with regard to the reduction of cancer cell viability or tumour size after administrating determined dosage LAB, but a few ones mentioned which compounds cause these effects (Zhang *et al*., 2005, Kato *et al*., 1994).

In the present study, both culture supernatant and WSPES were proved to be effective on two human cancer cell lines by using SRB assay shown in the table 5 and figure 6. These extracted samples of *L. rhamnosus* PN04 potently inhibited the viability of each cell line (HeLa and Hep G2 cells). Furthermore, these results indicated

that water soluble polysaccharides may contributed to the anticancer effects of *L. rhamnosus* PN04.

CONCLUSION

The culture medium and WSPES derived from *L. rhamnosus* PN04 exert significant antioxidant activity, as well as anticancer activity, on two HeLa, Hep G2 cell lines. These polysaccharide components may be applied to various foods, and used as adjuncts in cancer trials. In future, the optimizing conditions for industrial scale production of WSPES fraction will be done, both samples need to be tested on healthy cell to strengthen the capable of effect to cancer cell lines of *L. rhamnosus* PN04.

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