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Determination of the Antioxidant Activities of Ethanol Extracts from *Lactobacillus rhamnosus* PN04

T.A. Dao Nguyen and H.K. Tu Nguyen*

School of Biotechnology, Hochiminh International University, Hochiminh city, Vietnam.

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The aim of the present study was to determine the capacity of producing antioxidant agents of *Lactobacillus rhamnosus* PN04. The ethanol precipitated culture of *L. rhamnosus* PN04 was studied for reducing power and DPPH radical scavenging activity on both supernatant and a 90% ethanol extract. The reducing power was increased in the stationary phase and decreased at the death phase. At 10mg/ml, the absorbance of water-soluble exopolysaccharide (WSPES) solution at the stationary phase showed the highest value at 0.0355, compared to ascorbic acid standard at concentration 671 μg/ml. This results indicated that both the supernatant (115.3×10^6 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 30.04 μg/ml and 5.455 μg/ml, respectively. Consequently, *L. rhamnosus* PN04 may be a potential natural antioxidant.

Key words: Antioxidant activities, Ethanol extract, *Lactobacillus rhamnosus* PN04.

Recently, many evidences suggest that most of diseases are due to the "oxidative stress" resulting from an imbalance between formation and neutralization of pro-oxidants. Oxidative stress is initiated by free radicals, which produced aerobic metabolism in the body, could 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 contributed to oxidative stress was 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). Human cells protected themselves against free radical damage by enzymes such as superoxide dismutase and catalase, or compounds such as ascobic acid, tocopherol and glutathione (Niki, 1994). However, these protective mechansims occurred by various pathological processes. Therefore, 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. 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 toxicities have steadily been increasing.

* To whom all correspondence should be addressed.
Tel.: 084-8-37244270; Fax: 084-8-37242195;
E-mail: nhktu@hcmiu.edu.vn

Lactic acid bacteria such as *L. rhamnosus* is important microorganisms in a healthy human microbiologic, environment (Khalid *et al*., 2011; Macfarlane *et al*., 2002). *L. rhamnosus* are beneficial microorganisms, which have been associated with several probiotic effects in both humans and animals.

Recently, many research studies have focused on antioxidant properties as protective adjuncts against a host of diseases (Yang *et al*., 2001; Iwai *et al*., 2004). Numberous reports have indicated that both LAB and fermented milk exert antioxidant effects (Biffi *et al*., 1997; Lee *et al*., 2004).

Until recently, only a few laboratories had focused on the antioxidant effects of lactic acid bacteria, although several recent studies reported that components of *Lactobacillus* could be alleviative oxidative stress (Lin and Chang, 2000; Kullisaar *et al*., 2002). The objective of this study was to determine, in an in vitro context, the antioxidant properties of supernatant medium and polysacharides of *Lactobacillus rhamnosus* PN04.

MATERIALS AND METHODS

Culture *Lactobacillus rhamnosus* PN04

The bacteria was sub-cultured into 10 ml MRS and incubated at 37°C for 24 h. The resulting culture was used to inoculate a 100 ml MRS. The culture of *Lactobacillus rhamnosus* PN04 were incubated for 40 h at 37°C in aerobic condition. The culture was centrifuged (10000 rpm for 15 min, at 4°C).

Culture *L. rhamnous* PN04 at difference growth phase

L. rhamnous 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 (OD) measurement at wavelength of 600 nm was measured 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 collected at different growth phases were centrifuged at 10000 rpm for 30 min to separate the cell from the broth. The supernatant was precipitated with three times volume of 4°C cool absolute ethanol (EtOH). After overnight, the precipitant was collected by centrifugation 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. The WSPES stocks were filtered through a 0.22 µm pore-size filter (Millipore, Bedford, Mass.) and stored frozen at -80°C till used. The procedure used for EPS quantitation was based on that described by Dubois (Dubois *et al*., 1956) . The collected different phase samples were diluted 20 times in volume. Then, the total carbohydrate of sample solutions were measured colorimetrically, after addition of phenol solution (5 %w/v, 1 ml) and concentrated sulfuric acid (5 mL). This was left at 70°C for 20 minutes, mixed then placed in a cool water bath at 10°C for a further 10 minutes. The absorbance was measured spectrophotometrically at 490 nm against a blank (deionised water) and compared to a graph generated from the results obtained for a series of D-glucose standards.

Determination of antioxidant activity

Reducing power

For the assay of the reductive ability we investigated the Fe^{3+}- Fe^{2+} transformation in the presence of the samples using the Oyaizu (Oyaizu, 1986) method with a slight modification. Samples of 0.5 ml, 1 ml pH 6.6 phosphate buffer and 1ml 1% $K_3Fe(CN)_6$ were incubated at 50°C for 20 min. After incubation, 1 ml 10% trichloroacetic acid (TCA) was added to the mixture followed by centrifugation at 650 x g for 10 min. The upper layer (3ml) was mixed with 0.25 ml fresh 0.1% $FeCl_3$. The mixture was shaken and its absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power. A standard with ascorbic acid was made in a similar manner for comparison. All experiments were performed in triplicate.

DPPH radical scavenging activity

In order to perform the 2,2-diphenylpicrylhydrazyl (DPPH assay), 4.3 mg of DPPH was dissolved in 3.3 ml methanol and protected from light by covering the test tubes with aluminum foil. 150 microlit DPPH solution was added to 3ml methanol and absorbance was

taken immediately at 517 nm for control reading. 50 microlit of samples (10 mg/ml) as well as various concentration of standard ascorbic acid were taken and the volume was made uniformly to 150 microlit using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each 150 microlit 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.

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

RESULTS AND DISCUSSION

Study on *L. rhamnosus* PN04 growth phases

The growth of *L. rhamnosus* PN04 were measured and recorded (Tables 1). The best growth of *L. rhamnosus* PN04 was obtained after 21 h with OD 1.8. The growth increases slowly after incubated more 10h, giving an OD equaling to 1.89 (Table 1).

According to the growth of *L. rhamnosus* PN04, the different phase was decided at specific time shown in Table 1. The antioxidant activity tests were performed based on the culturing periods

Table 1. Weight of EPS at different phase of *L. rhamnosus* PN04

Growth phase	Weight of crude 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}

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

Table 2. Absorbance of D-glucose at different concentration using phenol/sulfuric acid assay

D-glucose standard						
Concentration (mg/ml)	0	0.2	0.4	0.6	0.8	1
Absorbance	0	0.91	1.55	2.08	2.67	3

Table 3. The percentage of total carbohydrate in crude EPS produced from cell-free supernatant *L. rhamnosus* PN04 at different growth phase

Growth phase	OD total carbohydrate (mg/ml)	Concentration of crude EPS(%w/w)	Total carbohydrate content
Early exponential phase	1.791	10.6	23.82%
Late exponential phase	2.399	14.48	28.67%
Stationary phase	2.345	16.34	31.12%
Death phase	2.659	16.44	32.81%

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

to know which phase gave the highest activity so that large scale will be done so far.

Preparation of polysaccharides

There have been many publications that have developed the procedure for isolating EPS. Most of these methods commonly use solvent such as acetone (Vincent *et al*., 2001; Lemoine *et al*., 1997; Lemoine *et al*., 1997) or ethanol (Shihata *et al*., 2002; Harding *et al*., 2005; Rodriguez *et al*., 2008) to precipitate the EPS. Since 2001, ethanol appears to be the solvent of choice and has been used in the majority of EPS precipitations.

From results showed in the table 1, the crude EPS were collected at four different phases. The crude EPS fractions were determined as in table 1 and Figure 1.

Although there was less significant difference between growth phases, the weight

Table 4. Reducing power capacity of ascorbic acid

	Absorbance 700 nm					
Concentration (µg/ml)	0	200	400	600	800	1000
Standard ascorbic acid	0	0.01	0.016	0.029	0.035	0.046

Table 5. Reducing power capacity of crude EPS of *L. rhamnosus* PN04

Growth phase	Reducing power of EPS	Concentration of ascorbic acid (µg/ml)
Early exponential phase	0.0284 ± 0.0007^{a}	569
Late exponential phase	0.0304 ± 0.0008^{b}	609
Stationary phase	0.0335 ± 0.0005^{c}	671
Death phase	0.0189 ± 0.0005^{d}	379

Table 6. DPPH scavenging activity of ascorbic acid

		Concentration (mg/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 7. DPPH scavenging activity of cell-free supernatant of *L. rhamnosus* PN04

Growth phase	% DPPH scavenging activity of supernatant	Concentration of ascorbic acid (mg/ml)
Early exponential phase	92.87 ± 0.075^{a}	28.71
Late exponential phase	94.28 ± 0.046^{b}	29.82
Stationary phase	95.19 ± 0.062^{c}	30.06
Death phase	94.91 ± 0.075^{d}	29.89

Table 8. DPPH scavenging activity of EPS of *L. rhamnosus* PN04

Growth phase	% DPPH scavenging activity of supernatant	Concentration of ascorbic acid (mg/ml)
Early exponential phase	35.68 ± 1.191^{a}	-
Late exponential phase	36.62 ± 0.075^{b}	-
Stationary phase	52.86 ± 0.133^{c}	5.46
Death phase	51.45 ± 0.112^{d}	4.64

(-): Not Value

at stationary phase is obtained highest amount (0.0525 g/5ml). That might also due to the best growth and *L. rhamnosus* PN04 EPS biosynthesis. The carbohydrate content of the dried exopolysaccharide for each batch was determined using a procedure based on that described by Dubois (Dubois *et al*., 1956). The method used was a colorimetric test for sugars and polysaccharides, where an orange colour was produced when carbohydrates were treated with a phenol/sulfuric acid solution. The intensity of this colorationis directly proportional to the amount of sugar present. The crude EPS was used to determine the carbohyrdrate contents based on the D-glusose standard curve (Table 2 and Figure 2). The carbohydrate contents were focused because of carbohydrate as hexasaccharide could be anticancer agent (Bharadwaj *et al.,* 2007; George *et al.,* 2006). EPS derived from LAB play crucial role in improving the rheology, texture, mouth feel of fermented food formulations and conferring beneficial physiological effects on human health, such as antitumour activity, immunomodulating bioactivity and anticarcinogenecity (Doleyres *et al*., 2005). LAB can also produce a variety of functional oligosaccharides. Oligosaccharides have huge industrial applications as prebiotics, nutraceuticals, sweetners, humectants, drug against colon cancer, immune stimulators etc. (Remaud *et al*., 2000).

The carbohydrate highly obtained in stationary phase till death phase (16.34 - 16.44 mg/ml culture) or 31.12 % - 32.81 % in EPS (Table 3). This indicated that the increasing weight of

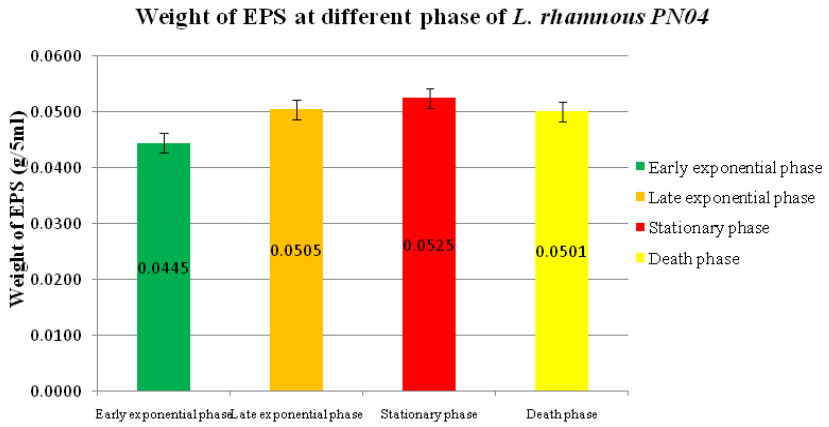

Fig. 1. Weight of crude EPS at difference phase *L. rhamnosus* PN04

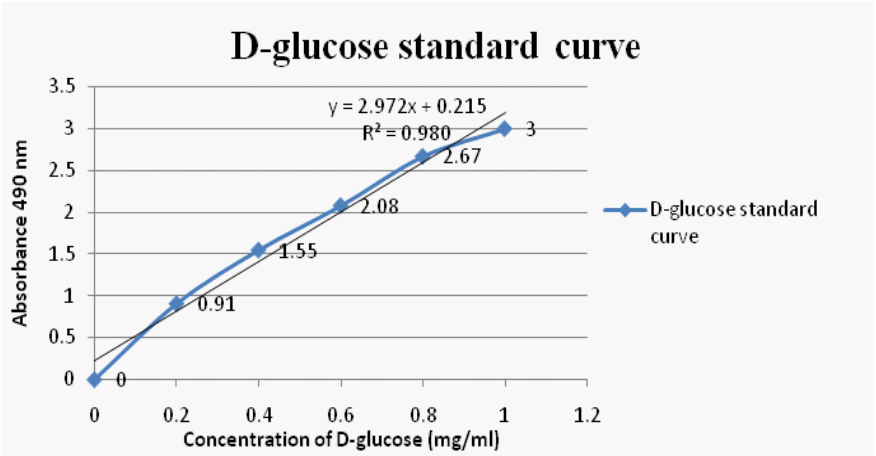

Fig. 2. Calibration curve for D-glucose standard curve using phenol/sulfuric acid assay

carbohydrate in EPS depended on the growth phase.

Moreover, the results show that the solid content is not only due to carbohydrate and other material must be present. The crude EPS samples need to study protein, DNA content and non-carbohydrate components so far.

Determination of antioxidant activity

Reducing power

The reductive ability is a significant indicator for its potential antioxidant activity. The reductive ability might be because of a hydrogen-donating ability (Shimada *et al*., 1992) and is generally associated with the presence of reductones (Pin-Der, 1998).

As illustrated in table 5 and figure 4, Fe^{3+} was tranformed to Fe^{2+} in the presence of *L. rhamnosus* PN04 extract and the reference compound ascorbic acid to measure the reductive capacity (Table 4 and Figure 3). At 10mg/ml, the absorbance of EPS at the four growth phases were 0.0284, 0.0304, 0.0335, 0.0189 compared with ascorbic acid at concentration 569, 609, 671, 379 µg/ml respectively. These results indicated that the maximun activity is shown at the stationary phase of the growth stage of *L. rhamnosus* PN04 than other phases. All samples were positive this indicated that in crude EPS of *L. rhamnosus* PN04 contained antioxidant agents.

DPPH radical scavenging activity

To confirm the ability of antioxidant activities of crude EPS and cell-free supernatant, DPPH methods were performed and results shown in table 7, table 8 and figure 5, figure 6. The ascorbic acid was considered as standard (Table 6 and Figure 4).

The DPPH radical inhibition assay is a widely used and comparably easy method to evaluate antioxidant activity. Because DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecule, which

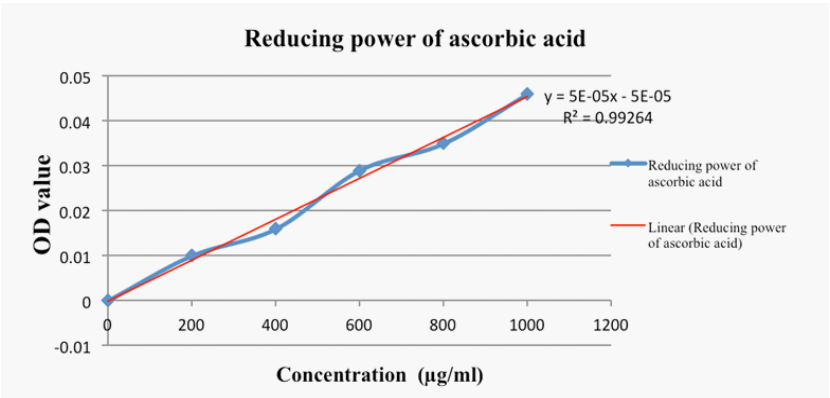

Fig. 3. Reducing power assay capacity of ascorbic acid

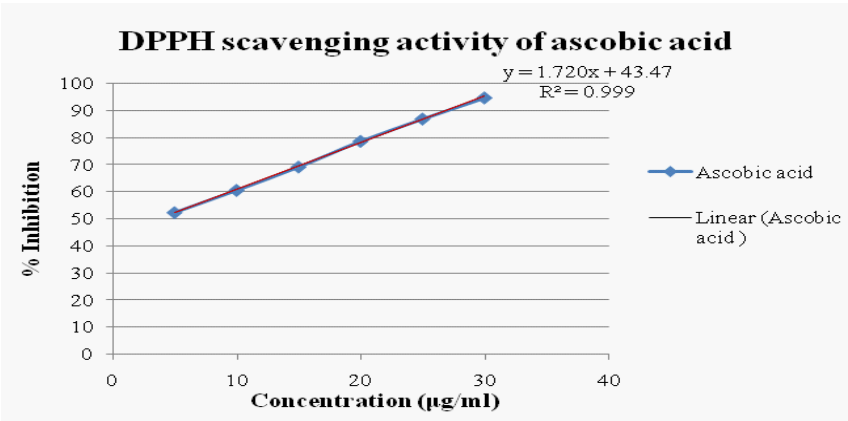

Fig. 4. DPPH scavenging activity of ascorbic acid

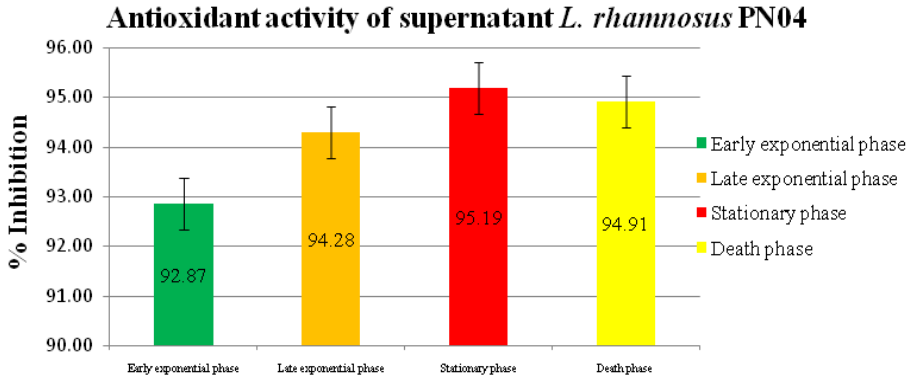

Fig. 5. DPPH scavenging activity of cell-free supernatant of *L. rhamnosus* PN04

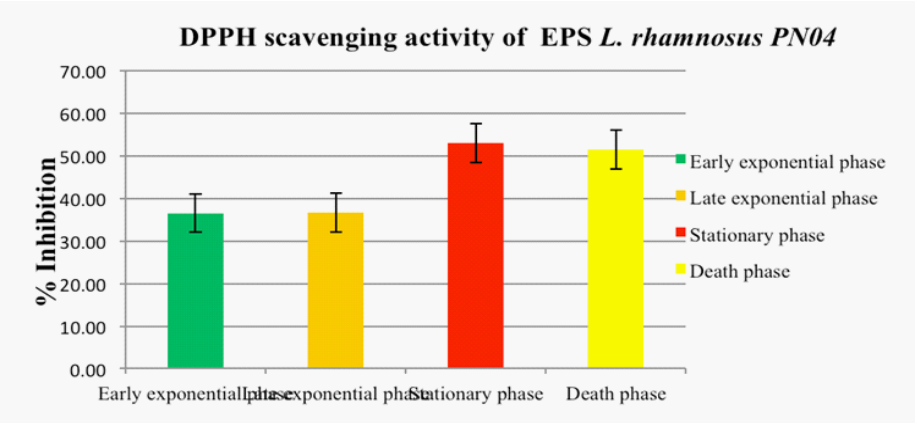

Fig. 6. DPPH scavenging activity of crude EPS of *L. rhamnosus* PN04

produces a violet solution in methanol. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent (Halliwell and Gutteridge, 2007).

DPPH radical is scavenged by antioxidants through the donation of photon forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration indicates that the free radical scavenging potentials of the antioxidant activity by their hydrogen donating ability. The electrons become paired off and solution loses colour electron.

Both the culture supernatant (10^8 cfu/ml) and 10 mg/ml EPS (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 30.06 µg/ml and 5.46 µg/ml respectively. As evident from these results, there were noticeable variability in the antioxidant activity of samples. And the antioxidative activity exhibited by the culture supernatant was more pronounced than that of the EPS fraction.

CONCLUSION

The study indicated that L. *rhamnosus* PN04 produced the significant amount of antioxidant agents. Therefore, this bacteria is a potential source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the phytoconstituents responsible for the antioxidant activity of *L. rhamnosus* PN04 should be studied.

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REFERENCES

1. Ames, B. N. Dietary carcinogens and anticarcinogens oxygen radicals and degenerative diseases. *Science*, 1983; **221**(4617): 1256-1264.
2. Biffi, A., Coradini, D., Larsen, R., Riva, L., & Di Fronzo, G. Antiproliferative effect of fermented milk on the growth of a human breast cancer cell line, 1997; **28**(1): 93-99
3. Branen, A. L. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *Journal of the American Oil Chemists' Society*, 1975; **52**(2): 59-63.
4. Büyükokuroðlu, M. E., GülçIn, I., Oktay, M., & Küfrevioðlu, O. I. In vitro antioxidant properties of dantrolene sodium. *Pharmacological research: the official journal of the Italian Pharmacological Society*, 2001; **44**(6): 491-494.
5. Chen, X. H., Xia, L. X., Zhou, H. B., & Qiu, G. Z. Chemical Composition and Antioxidant Activities of Russula griseocarnosa sp. nov. *Journal of agricultural and food chemistry*, 2010; **58**(11): 6966-6971.
6. Devasagayam, T., Tilak, J. C., Boloor, K. K., Sane, K., Ghaskadbi, S., & Lele, R. Free radicals and antioxidants in human health: current status and future prospects. *Japi*, 2004; **52***:* 794-804.
7. Diaz, M. N., Frei, B., Vita, J. A., & Keaney Jr, J. F. Antioxidants and atherosclerotic heart disease. *New England Journal of Medicine*, 1997; **337**(6): 408-416.
8. Gey, K.F. The antioxidant hypothesis of cardiovascular disease: epidemiology and mechanisms. *Biochem. Soc. Trans*. 1990; **18:** 1041-1045.
9. Goyal, A. K., Middha, S. K., & Sen, A. Evaluation of the DPPH radical scavenging activity, total phenols and antioxidant activities in Indian wild Bambusa vulgaris Vittata methanolic leaf extract. *Journal of Natural Pharmaceuticals*, 2010; **1**(1): 40-45.
10. Gülçin, I., Oktay, M., Küfrevioðlu, Ö. Ý., & Aslan, A. Determination of antioxidant activity of lichen Cetraria islandica (L) Ach. *Journal of Ethnopharmacology*, 2002; **79**(3): 325-329.
11. Gutteridge, J. M., & Halliwell, B. Invited review free radicals in disease processes: a compilation of cause and consequence. *Free Radical Research*, 1993; **19**(3): 141-158.
12. Halliwell, B. How to characterize an antioxidant: an update. *In Biochem Soc Symp*, 1995; **61**: 73-101.
13. Halliwell, B., & Gutteridge, J. M. Free radicals in biology and medicine, *Oxford university press*, 2007; 30-74.
14. Iwai, K., Kishimoto, N., Kakino, Y., Mochida, K., & Fujita, T. In vitro antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans. *Journal of agricultural and food chemistry*, 2004; **52**(15): 4893-4898.
15. Jorgensen, L. V., Madsen, H. L., Thomsen, M. K., Dragsted, L. O., & Skibsted, L. H. Regeneration of phenolic antioxidants from phenoxyl radicals: an ESR and electrochemical study of antioxidant hierarchy. *Free radical research*, 1999; **30**(3): 207-220.
16. Khalid, K., Kiong, L. H., Chowdhury, Z. Z., & Khalid, K. Antimicrobial interaction of Lactococcus lactis subsp. lactis against some pathogenic bacteria. *International Journal of Biosciences (IJB)*, 2011; **1**(3): 39-44.
17. Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C., & Kilk, A. Two antioxidative lactobacilli strains as promising probiotics. *International journal of food microbiology*, 2002; **72**(3): 215-224.
18. Lee, J. W., Kim, E. H., Yim, I. B., & Joo, H. G. Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. *Journal of Veterinary Science*, 2004; **5**(1): 41-48.
19. Lin, M. Y., & Chang, F. J. Antioxidative effect of intestinal bacteria Bifidobacterium longum ATCC 15708 and Lactobacillus acidophilus ATCC 4356.*Digestive diseases and sciences*, 2000; **45**(8): 1617-1622.
20. Macfarlane, G. T., & Cummings, J. H. Probiotics, infection and immunity. *Current opinion in infectious diseases*, 2002; **15**(5): 501-506.
21. Mon, M. M., Maw, S. S., & Oo, Z. K. Quantitative determination of free radical scavenging activity and anti-tumor activity of some Myanmar herbal plants. *World Acad. Sci. Eng. Tech*, 2011; **75**: 524-530.
22. Niki, E., Shimaski, H., & Mino, M. Antioxidantism-free radical and biological defense.*Gakkai Syuppn Center, Tokyo*, 1994; *3*.
23. Oyaizu, M. Studies on products of browning reaction—antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese journal of nutrition,* 1986; **44**(6): 307-315.
24. Patel Rajesh, M., & Patel Natvar, J. In vitro antioxidant activity of coumarin compounds by

DPPH, Super oxide and nitric oxide free radical scavenging methods. *Journal of Advanced Pharmacy Education & Research*, 2011; **1**: 52-68.

25. Prakash, D., Suri, S., Upadhyay, G., & Singh, B. N. Total phenol, antioxidant and free radical scavenging activities of some medicinal plants. *International journal of food sciences and nutrition*, 2007; **58**(1): 18-28.
26. Shahidi, F., Janitha, P. K., & Wanasundara, P. D. Phenolic antioxidants. *Critical Reviews in Food Science & Nutrition*, 1992; **32**(1): 67-103
27. Smith, M. A., Richey, G. P. P., Sayre, L. M., Anderson, V. E., Beal, M. F., & Kowall, N. Test for oxidative damage in Alzheimer's. *Nature*, 1996; **382**: 120-121.
28. Squadrito, G. L., & Pryor, W. A. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radical Biology and Medicine*, 1998; **25**(4): 392-403.
29. Yang, C. S., Landau, J. M., Huang, M. T., & Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annual review of nutrition*, 2001; **21**(1), 381-406.
30. Yen, G. C., & Chen, H. Y. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry*, 1995; **43**(1): 27-32.