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Original Article

Antioxidant Properties of Exopolysaccharides Produced by an Indigenous Species of *Penicillium* BMSP-12

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ABSTRACT

Microorganisms are a good source of exopolysaccharides (EPS) which have been reported in different microbial types including bacteria, fungi, and algae. Exopolysaccharides are mainly known for high-value applications, and have received considerable research attention over recent decades due to environmental and human compatibility. Among many biological properties, they play an important role as free radical scavengers and antioxidants for the prevention of oxidative damage in living organisms. In present investigation the antioxidant properties of the EPS produced by an indigenous species of *Penicillium* has been evaluated *in vitro*. The crude EPS obtained was purified by DEAE-52 and sephadex G-100, which resulted in EPS as the major polysaccharide produced by *Penicillium* BMSP-7.The FTIR of the EPS was performed to get the information regarding type of functional groups present in its structure. The antioxidant properties analyzed in crude and purified EPS samples, revealed the crude EPS as the more potential antioxidant as compared to the purified forms especially the reducing power and the scavenging ability on superoxide radicals. Both these activities were comparable with that of the standard (ascorbic acid).

Keywords: Indigenous, *Penicillium* sp., Antioxidant, Exopolysaccharide

1. INTRODUCTION

The increasing significance of microbial exopolysaccharides in industrial and medical applications necessitated the exploration of newer organisms to study the interrelations between metabolic pathways and exopolysaccharides biosynthesis mechanism in order to control, enhance their productivity and to evaluate their biological properties like antioxidant property. However, in this regard only a few

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microbial exopolysaccharides have achieved to be used commercially [1]. Microorganisms, especially the fungi, are well known to have an abundance of antioxidant compounds like exopolysaccharides that have been shown to be effective in removing reactive oxygen species (ROS) such as superoxide, anions, hydrogen peroxide, and hydroxyl radicals from the body. ROS are strongly associated with cardiovascular disease, cancer and various neurodegenerative disorders. The main sources of free radicals are oxidation reactions of polyenoic fatty acids [2]. Antioxidants have become one among the most important topics in human nutrition because of high concentrations of free lipid radicals, both in food and in vivo after food ingestion [3].Trauma faced by people these days is either in the form of mental or physical stress due to the consumption of noxious food and drugs which leads to oxidative stress in the body. In normal aerobic respiration, reactive oxygen species (ROS) are generated in the body after stimulation from exogenous factors or a number of endogenous metabolic processes [4]. Reactive species like hydroxyl radical, superoxide anion and various lipid peroxides cause damage by reacting with biomolecules [5]. These free radical-initiated reactions are known to bring on a wide variety of pathological effects, such as carcinogenesis, atherosclerosis, DNA damage and degenerative processes associated with ageing [6, 7]. Antioxidants are the agents capable of neutralizing the deleterious effects of ROS [8]. To diminish the damage to the human body, antioxidants are commonly used in processed foods. Antioxidants could alleviate the oxidative damage of a tissue indirectly by increasing natural defenses of cell and directly by scavenging the free radical species [9]. Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been extensively developed and utilized in recent years. However, the unwanted side effects caused by these synthetic antioxidants are suspected to be responsible for liver damage and carcinogenesis and other ailments [9, 10, 11].

Thus, there is a need to exploit new natural sources of antioxidants with low cytotoxic effect. In this regard a number of exopolysaccharides obtained from microorganisms have been found to possess potent antioxidant activities. From past several decades fungi are of immense interest because they are biologically rich in active substances [12.] Exopolysaccharide is widely used macromolecule in the fields of medicine and industry [13] . The exopolysaccharides obtained from microorganisms, especially from fungi like *Morchella crassipes*, *Cordy cepssinensis* and *Aspergillus* sp Y16 etc. have already been reported to possess remarkable antioxidant activity [14, 15, 16]. Therefore, the objective of the present study was to determine the *in vitro* antioxidant potential of the exopolysaccharides produced by an indigenously obtained species of *Penicillium*BMSP-7.

2. MATERIALS AND METHOD

The fungal culture

The culture of *Penicillium* BMSP-7 was isolated from the rhizospheric soil sample collected from the Panjab University Campus, Chandigarh, India. The pure culture was maintained on potato dextrose agar (PDA) slants and stored at 4°C.

Exopolysaccharide Fermentation & Extraction

The fermentation for exopolysaccharides production was carried out as per Lima *et al.*, 2008 and Misaki *et al.*, 2009 [17, 18] in 200ml Richard's fermentation broth in 500 ml flask by inoculating with two mycelial discs(10mm diam.) of the test fungus, *Penicillium* BMSP-7. The flasks were incubated at 28±1ºC under shaking at 150rpm for seven days. The fermentation broth obtained after seven days of incubation was first filtered with Whatman No. 1 filter paper to separate the mycelial biomass and centrifuged at 10,000 rpm at 4°C for 20 min. to remove mycelial remnants (if any) left after filteration. The supernatant obtained after centrifugation was mixed with 5% TCA for deproteinization and kept overnight at 4°C. Next day the precipitated proteins were removed by centrifuging the broth as mentioned above and the supernatant was mixed with the 4 volume of ethanol (supernatant: ethanol = 1:4 v/v) and kept overnight again at 4°C. Next day the crude pellets of exopolysaccharides obtained after centrifugation was dialyzed against distilled water, concentrated, lyophilized and expressed in g/l.

Purification of Exopolysaccharides

Purification of crude exopolysaccharides was done by DEAE-52 anion exchange chromatography and Sephadex G-100 as perQiao *et. al*.,2009 [19] . Crude exopolysaccharides solution (10 mg/ml, 5 ml) was applied to a column of DEAE-52 (2.6 x 30 cm), and the column was stepwise eluted with 0.1, 0.3 and 0.5 M sodium chloride solutions at a flow rate of 60 ml/h. Eluate (10 ml/tube) was collected and the carbohydrates were determined by the phenol–sulfuric acid method (Dubois et. al., 1956). As results, two fractions of polysaccharides (F-1 and F-2) were obtained, concentrated, dialyzed and further purified through a column of Sephadex G-100 (2.6 x 50 cm). The resultant major fraction (EPS) was then concentrated, dialyzed and lyophilized for further study, respectively.

FTIR spectroscopy of EPS

FTIR analysis of EPS was done by grinding 2mg of EPS with 200mg of dry potassium bromide and then pressed into a1mm pellet for recording FTIR spectra on FTIR infrared spectrometer in the frequency range of 4000-400cm^{-1}.

Determination of antioxidant activities *in vitro* of EPS

Determination of reducing power

The reducing ability of EPS samples was determined as per method of Liu *et al.*, (2009) and Ozen (2010) with some modifications [21, 22]. 2.5ml of each test sample (0.05-1mg/ml) prepared in distilled water was mixed with 2.5ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5ml of potassium ferricyanide (1%, w/v) and incubated at 50°C for 20

minutes. After incubation, 2.5ml of TCA (10%, w/v) was mixed and the mixture was set to centrifugation at 5000 rpm for 10 minutes. 5ml of upper layer was mixed with 0.5ml of fresh $FeCl_3$ (0.1%, w/v), and absorbance was measured at 700 nm. Distilled water and ascorbic acid were used as the blank and control, respectively. Higher absorbance indicates greater reductive potential.

Determination of scavenging ability on superoxide radical

The superoxide radical scavenging activity was investigated by the method of Kong *et al*., (2010) and Liu *et al*., (2009) with some modifications [7, 21] . The superoxide anion was generated in 3ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 156 µM β-nicotinamide adenine dinucleotide (NADH), 52 µM nitroblue tetrazolium (NBT) and 20 µM phenazinemethosulfate (PMS). 1ml of each test sample (0.05-1mg/ml) was added and incubated at 25°C for 5 minutes. Distilled water and ascorbic acid were used as the blank and control, respectively. The absorbance of the mixture was measured at 560nm. The scavenging activity on superoxide radical was determined as (%) = $(1-A_{sample}/A_{blank})$ x 100.

Determination of hydroxyl radical scavenging activity

The method of Jin *et al*., (1996) and Liu *et al*., (2009) with some modifications [23, 21] was used to determine the hydroxyl radical scavenging activity. The hydroxyl anion was generated in the mixture of 1ml of 0.75 mM 1,10-phenanthroline, 1.5ml of 0.15 M sodium phosphate buffer (pH 7.4), 1ml of 0.75 mM $FeSO_4$ and 1ml of H_2O_2 (0.01%, v/v). 1ml of each test sample (0.05-1mg/ml) was mixed and incubated at 37°C for 30 minutes. Distilled water and ascorbic acid were used as blank and control, respectively. Finally absorbance was measured at 536nm. The scavenging activity on hydroxyl radical was determined as (%) = $(A_{sample} - A_{blank}) /(A_0 - A_{blank})$ x 100, where A_0 was the absorbance of the distilled water instead of H_2O_2 and sample in the assay system.

Statistical analysis

Data were expressed as mean ± S.D. (n = 3). The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design. Graph pad prism (GPP Version 6.0) was used for the statistical analysis of the experimental data. The statistical differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Extraction and purification the exopolysaccharide

Crude exopolysaccharides obtained as a result of fermentative production using an indigenous species of *Penicillium* BMSP-7 was purified firstly by separating through an anion-exchange chromatography. The DEAE-52 cellulose was used as an anion exchanger in the column from which two independent elution peaks (F1 and F2) were detected by the phenol-sulfuric acid assay (**Fig.1a**). Both the fractions were collected, concentrated and subjected to gel filtration chromatography using Sephadex G-100. As a result, each fraction generated one single elution peak termed EPS and EPS1, respectively (**Fig. 1b & 1c**). The recovery rates of EPS and EPS1 were 50.0 % and 17.6% respectively. The results indicated EPS as the major polysaccharide produced by *Penicillium* BMSP-7 and thus it was purified, dialyzed, concentrated, lyophilized and further subjected to structure analysis by FTIR to get the information regarding functional groups and evaluated for their antioxidant efficiency against ascorbic acid.

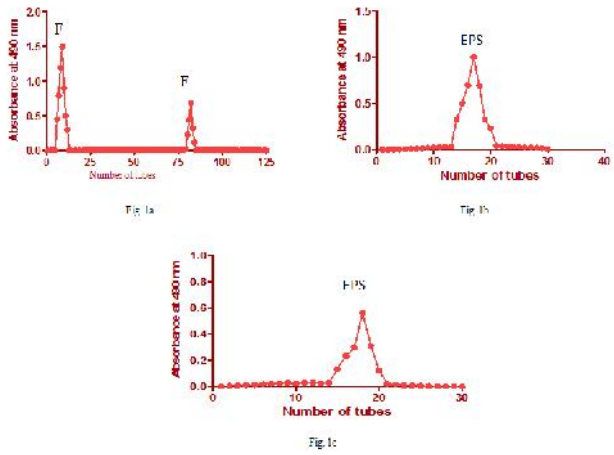

Fig 1: 1a) Stepwise elution curve of crude EPS on DEAE cellulose-52 column, confirming the presence of two different types of EPS peaks F1 & F2.Total 125 fractions were taken (25 each) with Phosphate buffer saline, 0.1M, 0.3M, 0.5M, 0.7M NaCl; 1b & 1c): Elution curve of exopolysaccharide fractions EPS and EPS1 obtained from F1 and F2 respectively on Sephadex G-100. EPS is the major EPS due to its high recovery rate.

Fourier transforms- infra red (FTIR) analysis of EPS

The functional groups of the purified EPS were recorded at the absorbance mode from 4000 to 400 cm^{-1} which revealed the presence of characteristic functional groups (**Fig 2**). The figure shows the IR spectra of EPS displaying intensity and broad band at around 3430 cm^{-1}, assigned to the hydroxyl groups (OH) stretching vibration. Signals at 2877 cm^{-1} were from the stretch vibration of CH group. Absorption bands at 1641 cm^{-1} assigned to an NH bending, signal at 1521 cm^{-1} assigned to NO group. A low intensity band at1456 cm^{-1} signifies CH_3 group. Signal at 1351 cm^{-1} attributed to C-O-C aryl alkyl asymmetrical group. Band at 1299 cm^{-1} assigned to esters. Signal at 952 cm^{-1} shows CH_2 group and bands from 675 to 870 cm^{-1} attributed to aromatic CH bending. The IR-spectrum, in our study, is in consistent with the results reported previously like Xiong *et al.* 2010 and Lin and Harichund 2011 [24, 25].

Antioxidant activities *in vitro* of EPS

Reducing power of EPS

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. In the reducing power assay, the yellow colour of test solution changes into various shades of green and blue colours depending on the reducing power of antioxidant samples.

Antioxidants are able to reduce Fe3+/ferricyanide complex to its ferrous form. Therefore, Fe^{2}+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm [30]. The result obtained were concentration-dependent that is with the increase in concentration, the reducing power of the test sample and ascorbic acid (control) also increased (**Fig3a**). At 1.2mg/ml, the reducing power of EPS was 0.911, which is near to the value of control. The results indicate that EPS have good reducing power and fulfill the requirement of an antioxidant. The reason may be some interactions and synergistic effects for antioxidant properties. It suggested that reductone-associated and hydroxide groups of EPS from *Penicillium* BMSP-2 can act as electron donors and can react with free radicals to convert them to more stable products.

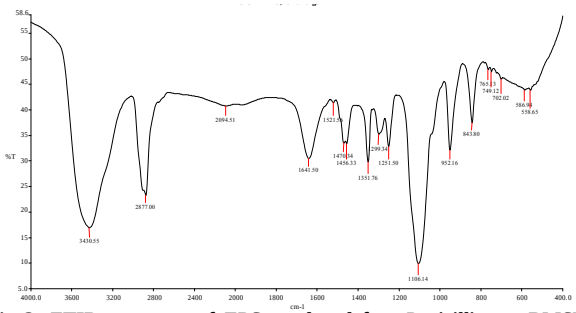

Fig 2: FTIR spectrum of EPS produced from*Penicillium*sp BMSP-7 showing the peaks of different functional groups which are present in structure of purified EPS.

Scavenging ability on superoxide radicals

Superoxide radicals were generated in a PMS/NADH system for being assayed in the reduction of NBT. Superoxide radicals would play important roles in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical and single oxygen, which induce oxidative damage in lipids, proteins and DNA [31]. **Fig. 3b**shows the scavenging effects of EPS and ascorbic acid (control) on the superoxide radicals. At a concentration of 0.2mg/ml, the scavenging effect of the EPS was 93.38%. The results were concentration-dependent as it revealed, with the increase in concentration the scavenging activity decreased. Scavenging activity of control was lower than the test, which shows remarkable result and EPS was found to exhibit stronger scavenging activity on superoxide radical. Although superoxide radical is relatively a weak oxidant in most organisms, it could produce hydrogen peroxide and hydroxyl radical through dismutation and other types of reaction and is the source of free radicals formed in vivo. These results indicate that EPS have strong scavenging activity on superoxide radical.

Scavenging ability on hydroxyl radicals

Hydroxyl radical is considered to be a highly potent oxidant, which react with most bio-macromolecules functioning in living cells and induce severe damage to adjacent biomolecules. Thus, removing hydroxyl radical is important for antioxidant defense in cell or food systems [32]. In present investigation, the hydroxyl radical, generated by the Fenton reaction in the system, was scavenged by the test EPS produced by *Penicillium* BMSP-2. The scavenging effects of EPS and ascorbic acid (control) as shown in **Fig. 3c** revealed a moderate scavenging effect against hydroxyl radical and were found directly proportional to the concentration of both the EPS and the control. The maximum scavenging activities of both the test EPS and the control at the maximum concentration of 1.2mg/ml was found to be 69%, and 92%, respectively.

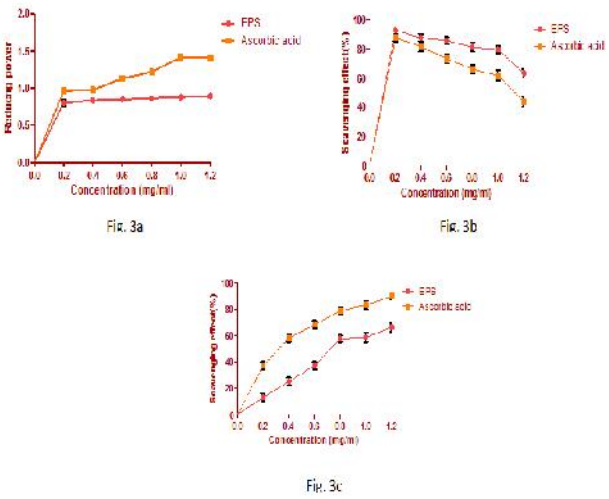

Fig 3: Antioxidant property of EPS obtained from *Penicillium*sp BMSP-7was determined by analyzing the followings: 3a: The reducing power of EPS and Ascorbic acid (Control); 3b: Scavenging activity on superoxide radical of EPS and Ascorbic acid (Control); 3c: Scavenging activity on hydroxyl radical of EPS and Ascorbic acid (Control). Data are presented as means ± SD of triplicates

4. CONCLUSION

In present investigation on *in vitro* antioxidant activities of the EPS obtained from an indigenous species of *Penicillium* indicated a strong antioxidant property. The antioxidant activity of EPS in terms of reducing power and the scavenging activity on superoxide radical were found to be comparable and even higher than the control (ascorbic acid) respectively. It might be due to the interference of the monosaccharide moiety and the molecular size of the pure EPS which affected or enhanced the antioxidant activity. The study is significant in two ways. Firstly, we have reported the antioxidant activity of the EPS produced by a tropical species of *Penicillium* and secondly, the activity was observed at lower EPS concentrations, which may find appropriate applications in food and pharmaceutical industries and may be effective even at smaller doses.

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6. REFERENCES

1. Sivakumar G, Medina-Bolivar F,Lay JJ, Dolan MC,Condori J, Grubbs SK, Wright SM,Baque MA, Lee EJ,Paek KY. Bioprocess and bioreactor: next generation technology for production of potential plant-based antidiabetic and antioxidant molecules.Curr. Med. Chem.,2011; 18(1): 79-90.
2. J Pokorny. Are natural antioxidants better – and safer – than synthetic antioxidants? Eur. J. Lipid Sci. Technol.2007; 109(8): 629-642.
3. Wang H, Liu YM, Qi ZM, Wang SY, Liu SX, Li X, Wang HJ, Xia XC. An overview on natural polysaccharides with antioxidant properties.Curr. Med. Chem.2013; 20(23): 2899-2913.
4. Sreelatha S, Padma PR. Antioxidant activity and total phenolic content of *Moringaoleifera* leaves in two stages of maturity. Plant Food Hum Nutr.2009; 64:303-311.
5. Bruschi F, Chiumiento L. Trichiella inflammator myopathy: host or parasite strategy?.Parasites & Vectors.2011; 4:42; 1756-3305.
6. Blander G, de Oliveira RM, Conboy CM, Haigis M, Guarente L. (2003) Superoxide dismutase 1 knock-down induces senescence in human fibroblasts. J BiolChem 2003; 278: 38966-38969.
7. Kong F, Zhang M, Liao S, Yu S, Chi J, Wei Z. Antioxidant activity of polysaccharide-enriched fractions extracted from pulp tissue of *Litchi ChinensisSonn.*Molecules.2010;15:2152-2165.
8. Pan Y, Chen Y, Li Q, Yu X, Wang J, Zheng J. The synthesis and evaluation of novel hydroxyl substituted chalcone analogs with *in vitro* anti-free radicals pharmacological activity and in vivo anti-oxidation activity in a free radical-injury Alzheimer's model Molecules. 2011; 18:1693-1703.
9. Salar RK, Dhall A. Antimicrobial and free radical scavenging activity of extracts of some indian medicinal plants. J Med Plants Res. 2010; 4(22):2313-2320.
10. Fathi H, Ebrahimzadeh MA. Antioxidant and free radical scavenging activities of *Hypericum perforatum*L.(st.John's wort). Int J Forest Soil and Erosion. 2013; 3(2):68-72.
11. Witschi H. Responses of the lung to toxic injury. Environ Health Perspect. 1990; 85:5-13.
12. .Crognale S, Bruno M, Fidaleo M, Moresi M, Petruccioli M. Production of β-glucan and related glucan-hydrolases by *Botryosphaeriarhodina*. J ApplMicrobiol. 2006; 102(3):860-71.
13. Kim HO, Yun JW.A comparative study on the production of exopolysaccharides between two entomopathogenic fungi *Cordycepsmilitaris* and *Cordycepssinensis* in submerged mycelial cultures. J ApplMicrobiol2005; 99:728-738.
14. He P, Geng L, Mao D, Xu C. Production, characterization and antioxidant activity of exopolysaccharides from submerged culture of *Morchellacrassipes*. Bioprocess Biosyst Eng. 2012; 35(8): 1325-32.
15. Yan JK, Wang WQ, Ma HL and WuJY.Sulfation and Enhanced Antioxidant Capacity of an Exopolysaccharide Produced by the Medicinal Fungus *Cordycepssinensis*. Mol. 2010; 18 (1):167-177.
16. Yin Y, ChenY, Mao W, Tao H, Zhu W, Qi X, Chen Y, Li H, Zhao C, Yang Y, Hou Y, Wang C, Li N. Structural characterization and antioxidant properties of an exopolysaccharide produced by the mangrove endophytic fungus *Aspergillus* sp. Y16. Biores. Technol. 2011; 102(17): 8179–8184.
17. Lima LF, Habu S, Gern JC, Nascimento BM, Parada JL, Noseda MD, Goncalves AG, Nisha VR, Pandey A, Soccol VT, Soccol CR. Production and characterization of exopolysaccharides produced by *Agaricus brasiliensis* in submerged fermentation. ApplBiochem and Biotechnol.2008; 151:283-294.
18. Misaki A, Kakuta M, Meah Y, Goldstein IJ. Purification and characterization of the α-1,3-Mannosylmannose-recognizing lectin of *Crocus vernus* bulbs. J Biol Chem. 2009; 272:25455-25461.
19. Qiao D, Hu B, Gan D, Sun Y, Ye H, Zeng X. Extraction optimized by using response surface methodology, purification and preliminary characterization of polysaccharides from *Hyriopsiscumingii*. Carbohydr Polym.2009; 76: 422-429.
20. Durmu N, Co Kuncelebi K, Kadioglu A, Beyazoglu O. Phenolic and sugar compositions of some *Hieracium L.* (Asteraceae) leaves in north east Anatolia. Bulg J Plant Physiol. 2002; 28(1-2):30-35.
21. Liu J, Luo J, Ye H, Sun Y, Lu Z, Zeng X. Production, characterization and antioxidant activities *in vitro* of exopolysaccharides from endophytic bacterium *Paenibacilluspolymyxa* EJS-3. CarbohydrPolym.2009; 78:275-281.
22. Ozen T. Antioxidant activity of wild edible plants in the Black sea region of Turkey. GrasasAceites. 2010; 61(1):86-94.
23. Jin M, Cai YX, Li JR, Zhao H. 1,10-Phenanthroline-Fe^{2+} oxidative assay of hydroxyl radical produced by H_2O_2/Fe^{2+} .ProgBiochem and Biophy. 1996; 23:553-555.
24. Lin J, Harichund C. Isolation and characterization of heavy metal removing bacterial bioflocculants. Afr. J. Microbiol. Res. 2011; 5: 599-607.
25. Xiong Y, Wang Y, Yi Y, Li Q, Wang H, Chen, He N. Production and Characterization of a novel bioflocculant from *Bacillus licheniformis.* Appl. Env. Microbiol.2010; 76: 2778-2782.
26. Chung YC, Chang CT, Chao WW, Lin CF, Chou ST.Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus*

subtilis IMR-NK1. J Agric Food Chem. 2002; 50:2454-2458.
27. Lung MY, Huang WZ.Antioxidant properties of polysaccharides from *Laetiporussulphureus* in submerged cultures.Afr J Biotechnol. 2009; 11(23):6350-6358.
28. Wickens A P. Ageing and the free radical theory.Respir Physiol. 2001; 128:379-391.
29. Kunwar A, Priyadarsini KI.Free radicals, oxidative stress and importance of antioxidants in human health. J Med Allied Sci. 2011; (2):53-60.
30. Wickens AP (2001) Ageing and the free radical theory. *Respiration Physiology* 128:379–391.
31. Chung YC, Chang CT, Chao WW, Lin CF and Chou ST.Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by Bacillus subtilis IMR-NK1. *Journal of Agricultural and Food Chemistry*.2002; 50: 2454–2458.
32. Aruoma OI.Free radicals, oxidative stress, and antioxidants in human health and disease. *Journal of the American Oil Chemists Society*.1998; 75:199–212.

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