Research Article
www.ijrap.net

FREE RADICAL SCAVANGING ACTIVITY OF *ARISAEMA LESCHENAULTII* BLUME

Jain Vinay[1]*and Panigrahi MK[2]
[1]ShriRam College of Pharmacy, Banmore, Morena, M.P., India
[2]Berhampur University, Berhampur, Orissa, India

Received on: 16/02/12 Revised on: 22/04/12 Accepted on: 11/06/12

***Corresponding author**
Email: jainvinay2007@rediff.com

ABSTRACT
Antioxidant profile has been studied by using different *in vitro* models. Study includes inhibition of oxygen derived free radicals (ODFR). From antioxidant studies, it was clear that ethanolic extracts of different parts of *A. leschanaultii* can play multidimensional role. The ethanolic extracts of *A. leschanaultii* showed anti-oxidant activity against DPPH, NO, peroxide and H_2O_2 radical scavenging activity with IC_{50} value of 0.178, 0.178, 0.180 and 0.251 mcg/ml respectively. Blume of *A. leschanaultii* showed anti-lipid peroxidation effects in liver homogenate. Ethanolic extracts can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions thus, act as reducing agents. Among the various extracts ethanolic extract of blume showed better antioxidant profile than aqueous extracts of *A. leschanaultii*.
KEYWORDS: DPPH, Nitric Oxide, Hydrogen Peroxide, Ascorbic acid.

INTRODUCTION

The many number of medicinal plants are used in the cellular and metabolic disease treatment such as diabetes, obesity and cancer etc. There are some speculations that the generation of free radicals inside the body in some physiological conditions is resulted in the cellular changes and development of cancer and this could be neutralized by the antioxidants from different medicinal plants. Several studies have shown that plant derived antioxidant neutraceuticals scavenge free radicals and modulate oxidative stress-related degenerative effects[1,2]. Free radicals have been implicated in many diseases such as cancer, atherosclerosis, diabetes, neurodegenerative disorders and aging[3, 4]. Previous research reports suggest that higher intake of antioxidant rich food is associated with decreased risk of degenerative diseases particularly cardiovascular diseases and cancer[5].

The free radical neutralizing property of several plants was reported by previous studies. The extracts from number of medicinal plants which are known to have some biologically active principles are used in ayurvedic preparations and these extracts are prepared in bulk for commercial purpose. In this present study we have measured antioxidant activity of various extracts of *Arisaema Leschenaultii* Blume employing various *in vitro* assay methods, such as scavenging activity of DPPH, superoxide radical, inhibition of microsomal lipid peroxidation and reducing power.

MATERIALS AND METHODS

Collections and Authentication of drug material

The plant material of *Arisaema Leschenaultii* was collected from the local areas of Bhopal, Pachmadi, Madhya Pradesh, India, in the month of August. A Voucher specimen (A/007/2006) was deposited at the Department of Pharmacognosy, Barkatullah University, Bhopal for future reference. Drug material was stored at room temperature in an airtight container.

Extraction

Plant material of *A. leschenaultii* was collected from different regions, thoroughly washed, and dried at 55ºC in an air dryer for 48 hour. Dried plant parts were powdered with Wiley Mill (Model 4276-M, Thomas Scientific, USA) to pass 20 mesh sieve and stored in sealed plastic bags. About 200 gm of powdered material was taken and extracted with different techniques of extraction (soxhlet extraction, percolation, maceration, sonication, homogenization and microwave extraction) using different solvents (petroleum ether, chloroform, acetone, ethanol, methanol and water). Each process was repeated thrice for complete extraction. After extraction, extracts were combined and evaporated to dryness *in vacuo*.

In vitro studies

Antioxidant Studies

Determination of Total Phenolic Content

Total phenolic content (TPC) of the ethanolic and aqueous extract of *A. leschenaultii* was determined by the method of Folin-Ciocalteu reaction using gallic acid as standard (Sigma Aldrich Company, USA). To 100 µL of extract (100 µg/mL), add 500 µL of (50%) Folin-Ciocalteu reagent followed by the addition of 1 mL of 20% Na_2CO_3 solution. A mixture was incubated at room temperature for 20 min and the absorbance was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram samples[6].

Preparation of Standard Solution of Gallic Acid

A stock solution of gallic acid was prepared by dissolving 0.5 mg of gallic acid accurately in 10 mL methanol. From this stock solution, standard solutions of 10-60 µg/mL were prepared by transferring aliquots (0.1 to 0.6 mL) `of stock solution and adjusting the volume up to 1 mL with methanol.

Calibration Curve of Gallic Acid

Standard solutions of gallic acid (10 µL) were applied in triplicate on HPTLC plate. The plate was developed in a solvent system toluene-ethylacetate-formic acid (7:14:0.1 v/v) for gallic acid at 25±2ºC temperature and 40% relative humidity up to a distance of 8 cm. After development, the plate was dried in air and scanned at 291 nm. The peak areas were recorded. Calibration curve was prepared by plotting peak area vs. concentration.

Determination of 1, 1-Diphenyl-2-Picryl Hydrazyl (DPPH) Radical Scavenging Activity

The antioxidant activity, based on scavenging of stable DPPH free radical was determined[7]. Different concentration of test sample was added to 3 mL of 0.004% methanol solution of DPPH. Absorbance at 517 nm was measured after 30 min and the percent inhibition activity was calculated as

DPPH Scavenged (%) =
(A cont. - A test) / (A cont.) x 100

Where, A cont. = Absorbance of control reaction
A test = Absorbance of test reaction
The antioxidant activity of the extract was expressed as IC_{50}.

Determination of Nitric Oxide (No) Radical Scavenging Activity

Nitric oxide (NO) was generated from sodium nitropruside (SNP) and measured by Greiss reaction. SNP in aqueous solution at physiological pH instinctively generates NO, which intermingles with oxygen to generate nitrite ions that can be anticipated by Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). Scavengers of NO with oxygen leading to reduced production of NO. SNP (5 mM) in phosphate buffer saline (PBS) was mixed with different concentration of (100-500 µg/mL) drug dissolved in suitable solvent and incubated at 25ºC for 150 min. The above samples were reacted with Greiss reagent. The absorbance of chromophore created during diazotization of nitrite with sulphanilamide and following coupling with naphthyl ethylene diamine was read at 546 nm and referred to the absorbance of standard solution of potassium nitrite treated in the same way with Greiss reagent[8].

Superoxide Anion (O^{2-}) Radical Scavenging Activity

Measurement of superoxide anion (O^{2-}) scavenging activity of extracts was based on the method described with slight modification[9,10]. Superoxide radicals were generated non-enzymatically in Phenazine methosulphate – Nicotinamide adenine dinucleotide phosphate (PMS–NADH) systems by the oxidation of NADH and assayed by the reduction of nitro blue Tetrazolium (NBT). The superoxide radicals were generated in 1 mL of Tris-HCl buffer (16 mM, pH 8.0) containing NBT (50 µM) solution and NADH (78 µM) solution. The reaction was started by adding PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25ºC for 5 min and the absorbance at 560 nm was measured against blank samples using UV spectrophotometer. Decrease absorbance of the reaction mixture indicates increase superoxide anion scavenging activity. The percentage inhibition (% I) of superoxide anion generation was calculated using the following formula:

(%) I = (A0 – A1) / (A0) x 100

Where A0 was the absorbance of the control and A1 was the absorbance of extract and the standard compound.

Determination of Reducing Power

Sample solutions containing 100-500 µg extracts were prepared from the stock solution. To 1 mL aliquot of the extract, 1 mL of 0.2 M phosphate buffer pH 6.6 and 1 mL of 1% (w/v) $K_3Fe(CN)_6$ was added. The mixture was incubated at 50°C for 20 min. 10% w/v of trichloroacetic acid (1 mL) was added and the resulting mixture was centrifuged at 3000 rpm for 10 min. The supernatant (1 mL) was taken to which 1 mL of distilled water and 0.2 mL of 0.1% (w/v) $FeCl_3$ solution was added. The absorbance was measured at 700 nm using UV–Vis spectrophotometer (Perkin-Elmer, USA.). The reducing power of α-tocopherol was also determined. Increase absorbance of the reaction mixture indicates increase reducing power[11-12].

Determination Of H_2O_2 Radical Scavenging Activity

The ability of extract to scavenge H_2O_2 was determined. A solution of H_2O_2 was prepared in PBS (pH 7.4). H_2O_2 concentration was determined spectrophotometrically by measuring absorption with extinction coefficient for H_2O_2 of 81 m^{-1} cm^{-1}. Extracts (100-500 µg/mL) were added to H_2O_2 solution (0.6 mL, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing PBS without H_2O_2 [13].

Lipid Peroxidation Assay

The inhibitory activity of kudingcha against mitochondrial lipid peroxidation was measured by thiobarbituric acid reactive substance (TBARS) method. The preparation of mitochondria and measurement of lipid peroxidation (LPO) was reported previously[14].

The liver was perfused with ice cold 0.15 M KCl via portal vein (KCl wash out blood from the tissue). The perfuse liver was isolated and 10% (w/v) homogenate was prepared using a tissue homogenizer under ice cold (0-4ºC) condition. The liver homogenate was used for the study of *in vitro* lipid peroxidation.

The mixtures containing 0.5 mL of homogenate, 1 mL of 0.15 M KCl and 0.5 mL of different concentrations of drug extracts were prepared. Lipid peroxidation was initiated by adding 100 µL of 1 mM ferric chloride. The reaction mixtures were incubated for 30 min at 37ºC. After incubation, the reaction was stopped by adding 2 mL of ice cold 0.25N HCl containing 15% trichloroacetic acid (TCA) and 0.38% thiobarbituric acid (TBA) and 0.2 mL of 0.05% butylated hydroxy toluene (BHT). The reaction mixture was heated for 60 min at 80ºC, cooled and centrifuged at 5000 g (≈6900 rpm) for 15 min. The absorbance of the supernatant was measured at 532 nm wavelength against a blank, which contained all reagents except liver homogenate and drug. Identical experiments were performed to determine the normal (without drug and $FeCl_3$) and induced (without drug) lipid peroxidation level in the tissue.

The percentage of anti-lipid peroxidation effect (% ALP) was calculated by the following formula:

$$\% \text{ ALP} = \frac{FeCl_3 \text{ OD - Sample OD}}{FeCl_3 \text{ OD - Normal OD}} \times 100$$

Table 1: Total phenolic content in *A. leschenaultii* Blume extracts

Samples	Phenolic content (mg GAE/100 gm sample)
EEAL	349.0 ± 0.05
AEAL	309.0 ± 0.01

All values are expressed as mean ± SEM (n=6)

Table 2: Free radical scavenging activity of *A. leschenaultii* Blume extracts

Free radical	IC_{50} value (mg/mL)		
	AEAL	EEAL	Vit-E
DPPH	0.234 ±0.05	0.178±0.02	0.15±0.05
Nitric oxide	0.183±0.01	0.178±0.05	0.121±0.03
Super oxide	0.477±0.001	0.180±0.001	0.147±0.02
H_2O_2	0.217±0.05	0.251±0.001	0.201±0.001
LPO (liver)	0.684±0.01	0.397±0.01	0.295±0.01

All values are expressed as mean ± SEM (n=6).
AEAL: Aqueous extract of *A. leschenaultii*; **EEAL:** Ethanolic extract of *A. Leschenaultia*

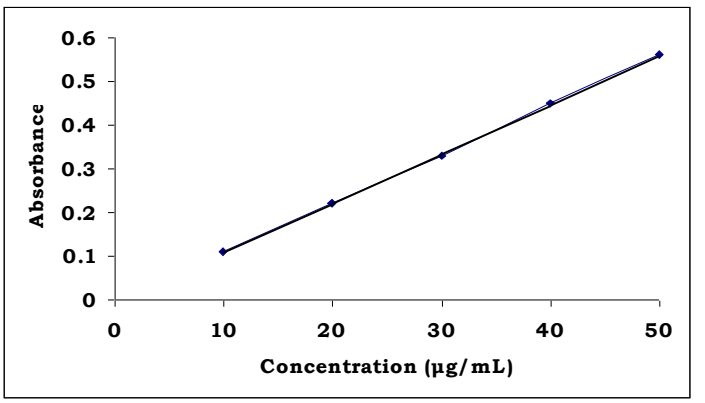

Figure 1: Calibration curve of gallic acid

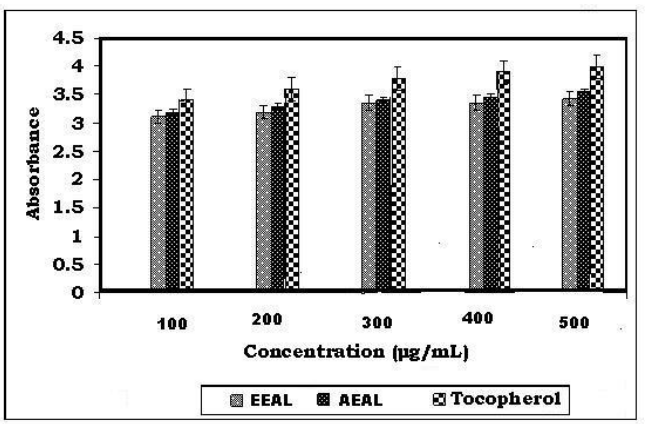

Figure 2: Reducing power activity of *A. leschenaultii* Blume extracts

RESULTS AND DISCUSSION

Antioxidant Activity

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Reactive oxygen species (ROS) are the by-products of oxygen involving metabolic reactions, which are controlled by a system of enzymatic and non-enzymatic antioxidants, which eliminate pro-oxidants and scavenge free radicals[15]. Once radicals form they can either react with another radical or another molecule by various interactions. The rate and selectivity of reactions of this type occurring depends on high radical concentration, delocalization of the single electron of the radical (thus, increasing its lifetime) and the absence of weak bonds in any other molecule present with which the radical could interact[16].

Total Phenolic Content

Antioxidant activity has been directly linked to the presence of phenolic moieties present in the molecular structure of natural antioxidants. Phenolic compounds are secondary metabolites that are synthesized in plants. They possess biological properties such as: antioxidant, anti-apoptosis, anti-aging, anti-cancer, anti-inflammatory, anti-atherosclerosis, cardiovascular protection, improvement of the endothelial function, as well as inhibition of angiogenesis and cell proliferation activity. Most of these biological actions have been attributed to their intrinsic reducing capabilities using delocalization of the single

electron of the radical and free radicals scavenging activity[17-18]. Hydrogen donating property of polyphenolic compounds is responsible for the inhibition of free radical induced lipid peroxidation[19].

Table 1 shows total phenolic content in different samples of *A. leschenaultii*. The total phenolic content of ethanolic extract of *A. Leschenaultii* blume (454 mg GAE/gm DW) was more than aqueous extract of blume. The linear equation (y=178.96x – 0.0204) for the calibration curve of gallic acid is depicted in Figure 1.

DPPH Radical Scavenging Activity

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It has characteristic absorbance maxima at 517 nm, widely used to evaluate the free radical scavenging effect of natural antioxidants[20]. Many researchers have reported positive correlation between free radical scavenging activity and total phenolic compound. DPPH radical scavenging activity increased with the increase of phenolic content [21-23].

Table 2 represents DPPH radical scavenging activity of ethanolic and aqueous extracts of *A. leschenaultii*. The different extracts of *A. Leschenaultii* significantly ($P<0.05$) scavenge DPPH radical in a concentration dependent manner. The scavenging activity of ethanolic extract of *A. Leschenaultii* blume was more than aqueous extract of *A. Leschenaultii* blume with IC_{50} value of 0.178 mg/mL which was significant as compared to α-tocopherol (Vit E) 0.151 mg/mL.

Nitric Oxide Radical Scavenging Activity

Reactive oxygen species (ROS) like superoxide ion may react with NO, resulting in the formation of reactive nitrogen species (RNS) such as NO_2 and N_2O_4. Both ROS and RNS cause cellular damage[24]. Nitric oxide radical scavenging activity of ethanolic and aqueous extracts of *A. leschenaultii* is presented in Table 2. Ethanolic and aqueous extracts of *A. leschenaultii* decreased the release of nitric oxide (NO) by sodium nitropruside (nitric oxide donor) in a dose dependent manner. Ethanolic extract of blume of *A. leschenaultii* showed higher NO scavenging activity (IC_{50} 0.178 mg/mL) as compared to aqueous extract of blume.

Superoxide Radical Scavenging Activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals[25]. In the PMS/NADH-NBT system, superoxide anions, derived from dissolved oxygen by PMS/NADH coupling reaction, reduce NBT. The decreased absorbance at 560 nm indicates the consumption of superoxide anions in the reaction mixture. Superoxide radical scavenging activity of ethanolic and aqueous extracts of *A. leschenaultii* is presented in Table 2. From the investigations, it was found that the ethanolic extracts of *A. leschenaultii* inhibit superoxide radicals in a dose dependent manner. The ethanolic extract of *A. leschenaultii blume* exhibited maximum superoxide scavenging activity (IC_{50} value 0.180 mg/mL).

Reducing Power Activity

Antioxidants can be reductants and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other. The reducing capacity of a compound can be measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4\,[Fe(CN^-)_6]_3$, which has a strong absorbance at 700 nm. An increase in absorbance of the reaction mixture would indicate an increase in reducing capacity due to an increase in the formation of the complex. There are a number of assays designed to measure overall antioxidant activity or reducing potential, as an indication of a host's total capacity to withstand free radical stress[26].

Figure 2 shows reducing power activity of ethanolic and aqueous extracts of *A. leschenaultii*. The reducing power of the ethanolic extracts of *A. leschenaultii* and the reference compound, α-tocopherol increased steadily with concentration. The reducing power (absorbance at 700 nm) of ethanolic extract of *A. leschenaultii blume* and α-tocopherol was 3.65 and 4.02. This indicated that ethanolic extracts can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions. The results were significant as compared to control ($P<0.05$).

H_2O_2 Radical Scavenging Activity

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes such as superoxide dismutase. It is known that H_2O_2, either directly or indirectly via its reduction product OH^-, acts as a messenger molecule in the synthesis and activation of inflammatory mediators. It can cross membranes and may slowly oxidize a number of compounds. It is used in the respiratory burst of activated phagocytes. It is known that H_2O_2 is toxic and induces cell death *in vitro*. Hydrogen peroxide can attack many cellular energy producing systems[27]. Hydrogen peroxide radical scavenging activity of different extracts of *A. leschenaultii* is presented in Table 2. The different extracts of *A. leschenaultii* showed H_2O_2 radical scavenging activity. Ethanolic extract of blume of *A. leschenaultii* significantly inhibited peroxide radical with IC_{50} value 0.251 mg/mL.

Lipid Peroxidation Assay

Malondialdehyde (MDA) is the major product of lipid peroxidation, along with the other aldehydes and ketones. These are commonly known as thiobarbituric acid reactive substances (TBARS). These are used to study the lipid peroxidation process in rat liver homogenates. Determination of the lipid peroxides was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic conditions. It is known that a cleavage product of lipid peroxidation accumulates in nervous system, cardiovascular system and muscle fibres[28]. Lipid peroxidation activity of different extracts of *A. leschenaultii* is presented in Table 2. The ethanolic extract of *A. leschenaultii* showed maximum anti-lipid peroxidation effect in liver homogenate with IC_{50} value of 0.397 mg/mL. The result showed that inhibition of TBARS formation in rat liver homogenate increases with increasing concentration.

CONCLUSION

Antioxidant profile has been studied by using different *in vitro* models. Study includes inhibition of oxygen derived free radicals (ODFR). From antioxidant studies it was clear that ethanolic extracts of different parts of *A.*

leschanaultii can play multidimensional role. The ethanolic extracts of *A. leschanaultii* showed anti-oxidant activity against DPPH, O^{2-}, peroxide and NO radical. Blume of *A. leschanaultii* showed anti-lipid peroxidation effects in liver homogenate. Ethanolic extracts can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions. Thus, act as reducing agents. Among the various extracts ethanolic extract of blume showed better antioxidant profile than aqueous extracts of *A. leschanaultii*.

REFERENCES

1. Ames BN, Shigenaga MK and Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America. 1993; 90: 7915-7922.
2. Joseph JA, Shukitt-Hale B, Denisova NA, Bielinski D, Martin A, McEwen JJ and Bickford PC. Reversal of age related declines in neuronal signal transduction, Cognitive and Motor behavioral Deficits with Bluberry, spinach, or Strawberry Dietery Supplementation. J Neurosci. 1999; 19: 8114-8121.
3. Halliwell B and Gutteridge JM, Free Radicals in Biology and Medicine. Oxford University Press, 1999.
4. Yu BP, Cellular defenses against damage from reactive oxygen species. Physiol Rev. 1994; 76: 139-162.
5. *T*hatte U, Bagadey S, Dahanukar S. Modulation of programmed cell death by medicinal plants. [Review]. Cell Mol Biol. 2000; 46: 199-214.
6. Kujala TS, Loponen JM, Klika KD and Pihlaja K. Phenolic and betacyanins in red beet root (*Beta vulgaris*), root distribution and effects of cold storage on the content of total phenolics and three individual compounds. Journal of Agriculture and Food Chemistry. 2000; 48: 5338-5342.
7. Braca A, Tommasi ND and Bari LD. Antioxidant principles from *Bauhinia terapotensis*. Journal of Natural Products. 2001; 64: 892-895.
8. Green LC, Wagner DA and Glogowski J. Analysis of nitrate, nitrite and 15 (N) nitrate in biological fluids. Analytical Biochemistry. 1982; 126: 131-138.
9. Liu F, Ooi VEC and Chang ST. Free radical scavenging activity of mushroom polysaccharide extracts. Life Science. 1997; 60: 763-771.
10. Oktay M, Gulcin I and Kufrevioglu OI. Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Lebensmittel Wissenchaft und Technology. 2003; 36: 263-271.
11. Oyaizu M. Studies on product of browning reactions prepared from glucose amine. Japanese Journal of Nutrition. 1986; 44: 307-315.
12. Singh N and Rajini PS. Free radical scavenging activity of an aqueous extract of potato peel. Food Chemistry 2004;85: 611-616.
13. Ruch RJ, Cheng SJ and Klaunig JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogen. 1989; 10: 1003-1008.
14. Thuong PT, Kang HJ and Na MK. Anti-oxidant constituents from *Sedum takesimense*. Phytochemistry. 2007; 68: 2432-2438.
15. Paolo DM, Michael EM and Helmut S. Antioxidation defence systems: The role of carotenoids, tocopherols and thiols. American Journal of Clinical Nutrition. 1991; 53: 194-200.
16. Okezie IA. Characterization of drugs as antioxidant prophylactics. Free Radical Biology and Medicine. 1996; 20: 675-705.
17. Gil MI, Barberan FAT, Pierce BH, Holcroft DM and Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. Journal of Agriculture and Food Chemistry. 2000; 48: 4581-4589.
18. Han X, Shen T and Lou H. Dietary polyphenols and their biological significance. International Journal of Molecular Science. 2007; 8: 950-988.
19. Yen GC, Duh PD and Tsai CL. The relationship between antioxidant activity and maturity of peanut hulls. Journal of Agriculture and Food Chemistry. 1993; 41: 67-70.
20. Jao CH and Ko WC. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolyzate from tuna cooking juice. Fish Science. 2002; 68: 430-435.
21. Lu Y and Foo YL. Antioxidant and free radical scavenging activities of selected medicinal herbs. Journal of Life Science. 2000; 66: 725-735.
22. Oki T, Masuda M and Furuta S. Involvement of anthocyanins and other phenolic compounds in radical scavenging activity of purple fleshed sweet potato cultivars. Food and Chemical Toxicology. 2002; 67: 1752-1756.
23. Siriwardhana N, Lee KW, Kim SH, Ha JW and Jeon YJ. Antioxidant activity of Hizikia fusiformis on reactive oxygen species scavenging and lipid peroxidation inhibition. Food Science and Technology International. 2003; 9: 339-346.
24. Pacifici RE and Davies KJ. Protein, lipid and DNA repair system in oxidative stress: The free radical theory of aging revisited. Gerontology. 1991; 37: 166-180.
25. Dahl M and Richardson M. Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and amino acids. Journal of Dairy Science. 1978; 61: 400-407.
26. Wood LG, Gibson PG and Garg ML. A review of the methodology for assessing in vivo antioxidant capacity. Journal of Science and Food Agriculture. 2006; 86: 2057-2066.
27. Halliwell B, Gutteridge JMC and Amoma OL. The deoxyribose method, a simple test tube assay for the determination of rate constant for reactions of hydroxyl radicals. Analytical Biochemistry. 1987; 165: 215-219.
28. Nohl H. Involvement of free radicals in ageing: A consequence or cause of senescence. British Medical Bulletin. 1993; 49: 653-667.

Source of support: Nil, Conflict of interest: None Declared