Int J Pharma Res Health Sci. 2016; 4 (4): 1305-1310
DOI:10.21276/ijprhs.2016.04.11
L Srinivas *et al.*
CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

Potent Antioxidant Activity of a Protease Inhibitor-hayanin from the Seed Coats of Horse gram (*macrotylomauniflorum* (lam.) Verdc.)

Prabhu MS Lingaiah, Leela Srinivas *

Adichunchanagiri Biotechnology and Cancer Research Institute, B.G Nagara-571448, Karnataka, India

ARTICLE INFO

Received: 02 July 2016
Accepted: 20 Aug 2016

ABSTRACT

Objective: The proteinaceous protease inhibitors from the plant source have been widely reported and have many therapeutical applications. The present study is to evaluate the antioxidant potentiality of protease inhibitors (named as Hayanin) isolated from the seed coats of Horse gram.**Methods:** Protease inhibitor from Horse gram seed coat was isolated and the phytochemical analysis of the isolated extract was analyzed for Proteins, Total sugars, Polyphenols, Flavonoids, Ascorbic acid and α- tocopherol at different stages of purification process. The antioxidant activity of the protease inhibitor was studied, to achieve this several antioxidant assays were done such as DPPH radical scavenging, Lipid peroxide inhibition, Hydroxyl radical scavenging, Superoxide radical scavenging and Reducing power potentiality. **Results:** The isolated protein with molecular mass of ~26kDa, displayed strong protease inhibitory activity, the proximate analysis shows that there was high concentration of protein as compared to negligible amounts of other phytochemicals. Hayanin at 50µg concentration inhibited Lipid peroxide inhibitory activity by 85 % and Hayanin indicated increased Reducing power potentiality in a dose dependent manner, effectively prevented Hydroxyl and Superoxide radicals at 75 % and 83% respectively. The protein – Hayanin has a remarkable potentiality in scavenging the DPPH radical by 88 %, which are comparable with the standard antioxidants such as BHA, Ascorbic acid and Curcumin.**Conclusion:** Hayanin has significant antioxidant activity against different ROS and these results encourage further studies as a potent therapeutic agent.

Keywords: Hayanin, Protease inhibitor, Antioxidant activity, Horse gram, Phytochemical analysis and *Macrotylomauniflorum.*

1. INTRODUCTION

Free radicals and other Reactive Oxygen Species (ROS) are formed due to oxidative stress [1], which have one or more unpaired electrons such as Superoxide ($O_2^{\cdot-}$), Hydroxyl ($^{\cdot}OH$), Peroxyl ($HOO^{\cdot}$), etc.,[2].These are also formed during the metabolic process, which affects mainly in the Lipid peroxidation [2], alteration of protein and nucleic acid

Corresponding author *
Dr. Leela Srinivas
Adichunchanagiri Biotechnology and Cancer Research Institute
BalagangadharanathaNagara
NagamangalaTaluk, Mandya-571448
Karnataka, India.
E-mail: prabhulingam6@gmail.com

structures [3, 4]. These free radicals lead to metabolic disorders or pathological problems of various diseases like Cancer, Asthma, Arthritis[5], Atherosclerosis, Parkinsons, Coronary heart diseases, Diabetes etc., [1, 6, 7, 8, 9, 10]. These ROS also induces several age related diseases and in the ageing process[11, 12]. Thus antioxidants play an important role in ameliorating the diseases caused by the oxidative stress induces free radicals[13, 14, 15]. Basically, all organisms have antioxidant systems that are able to act against free radicals such as Superoxide dismutase, Catalase, Peroxidase etc.,[5] and there are some proteins (Ferritin, Transferritin and Lactoferrin) helps in quenching the iron, ceruloplasmin and albumin involved in copper storage[16]. In case of adverse or excessive oxidative stress the normal antioxidant defensesystem does not provide complete protection against free radicals and thereby external supply of antioxidants to be done to counter those free radicals[5]. But, the excessive use of synthetic antioxidants (BHA, BHT) leads to adverse toxic effects at higher doses[17], which hinders their use in therapeutic applications.

To overcome this dietary sources have been selected as antioxidants which are non-toxic and easily available. Plants possess many bioactive components which can be used as antioxidants. Dietary fruits, vegetables and spices are good source of antioxidants (Curcumin, β-carotene, Ascorbic acid, Turmerin, etc.,) [18, 19]. Proteinaceous protease inhibitors isolated from plant source play a major role against many diseases by regulating or controlling endogenous proteases[20]. Most of the works on plant protease inhibitors aremainly concentrated on their potent insecticidal activities[20]. Protease inhibitors are extensively isolated from the families of Leguminosae, Gramineae and Solanaceae[21]which are otherwise an important source of food[22]. Horse gram **(***Macrotylomauniflorum* (Lam.) Verdc.**)**belongs of Leguminosae family used in the treatment of piles, pain, constipation, wounds, urinary tract infection, cough, edema, asthma, etc.,[23]. Horse gram has been extensively studies as protease inhibitors and the phenolic content shows antioxidant properties[24]. In this study, a proteinaceous protease inhibitor from Horse gram seed coat has been isolated[25]and tested for its antioxidant activity in different model system.

2. MATERIALS AND METHODS

2.1 Chemicals:

ButylatedHydroxy Anisole (BHA), Thiobarbutyric acid (TBA),Curcumin, 2,4- Di Nitro Phenyl Hydrazine(DNPH), Ethylene diamine tetra acetic acid (EDTA) and Ascorbic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Ferrous sulphate ($FeSO_4$), Ferric chloride ($FeCl_3$), Dimethyl Sulfoxide (DMSO), Nitro Blue Tetrazolium (NBT), 2,2- Diphenyl -1- picrylhydrazyl (DPPH), Bovine Serum Albumin (BSA), Superoxide Dismutase (SOD), Linoleic acid, Gallic acid and 3,5- Dinitro Salicylic acid (DNS) from Himedia Private Ltd. India. Hydrogen Peroxide (H_2O_2) and Folin-Ciocalteu reagentwere from SRL, India. All other chemicals unless otherwise mentioned were of analytical grade and procured from Merck India Pvt. Ltd.. Solvents were distilled before use.

2.2 Isolation and Purification of Hayanin:

Hayanin was purified according to the method of Prabhu MS Lingaiah and LeelaSrinivas (2015)[25]. Briefly, Horse gram seeds were washed and soaked in distilled water, then seed coat was removed, shade dried and powdered. 50g of seed coat powder was homogenized in 500 mL distilled water and the supernatant was subjected to ammonium sulphate precipitation (65%), centrifuged and the pellet was dissolved and dialyzed against distilled water. The dialyzed extract was loaded onto Sephadex G-50 column chromatography and the active peak was rechromatographed using the same parameters. The resulted active protein part with protease inhibitory activity having a molecular weight of ~26kDa (SDS-PAGE) was named as Hayanin[25].

2.3 Proximate Analysis:

2.3.1 Estimation of Protein:

The protein content was determined using Bradford's reagent[26] using Bovine Serum Albumin as standard. Different aliquots of the extract were made up to 100 µL with distilled water, to this 900 µL of Bradford's reagent was added and the color developed was read spectrophotometrically at 595nm (UV 1601, SHIMADZU, Japan).

2.3.2 Estimation of Total Sugars:

The total sugars were estimated by Dubois method [27]and Dextrose was used as standard. Different aliquots of Hayanin were made up to 1 mL with distilled water followed by the addition of 1 mL of 5% phenol and 5 mL of concentrated sulphuric acid. Orange colour developed was read at 520 nm. The sugar concentration was calculated accordingly.

2.3.3 Estimation of Total Phenols:

Total content of Phenols was determined by using Gallic acid as standard according to the method of Folin-Ciocalteu reaction[28] with minor modifications. Hayanin and the standard Gallic acid were dissolved in 500 µL of water and were mixed with 500 µL of 50% Folin-Ciocalteu reagent. The mixture was allowed to stand for 10 min. followed by the addition of 1 mL of 20% Sodium carbonate. Incubated at 37 ^{0}C for 10 min. and the absorbance of the supernatant were read at 730 nm. The total phenolic content was expressed as Gallic acid equivalents in milligrams.

2.3.4 Estimation of Flavonoids:

Flavonoid content was determined calorimetrically and the standard calibration curve was prepared using Quercetin[29]. 10 mg of Quercetin and Hayanin was dissolved in 80% ethanol and then diluted solution was separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min., the absorbance of the reaction mixture was measured at 415 nm

by using proper controls. The total flavonoid content was calculated accordingly.

2.3.5 Estimation of Ascorbic acid:

Ascorbic acid was determined according to the method of Sadasivam and Manickam[30], the aliquots of standard brominated ascorbic acid and Hayanin was taken and make up the volume to 3 mL with distilled water. Then 1 mL of DNPH and 1 mL of thio urea was added and incubated at 37^{0}C for 3 hrs. to form osazone to give orange red colour solution, this was measured at 540 nm. Dehydroascorbate alone reacts with 2, 4-dinitrophenyl hydrazine quantitatively and not the other reducing substances present in the extract.The total ascorbic acid content was determined accordingly.

2.3.6 Estimation of α-tocopherol:

α-tocopherol was estimated by using Emmerie reaction[31], aliquots of standard α-tocopherol along with Hayanin was taken and the made upto3 mL using chloroform. To this 1 mL of 2,2-dipyridyl reagent and 1 mL of ferric chloride was added, all tubes were incubated for 10 min., this is based on the reduction of α-tocopherol from ferric to ferrous ions which forms red colour with 2,2-dipyridyl and measured at 520 nm.

2.4 Antioxidant activity:

2.4.1 DPPH Radical Scavenging activity:

DPPH Radical scavenging activity was assessed according to the method of [32]. The α-tocopherol at various concentrations ranging from 0 to 100 µM was mixed with 1 mL of freshly prepared 0.5 mM DPPH ethanolic solution and 2 mL of 0.1M acetate buffer of pH 5.5. The resulting solutions were then incubated at 37 ^{0}C for 30 min. and measured calorimetrically at 517 nm. BHA, Curcumin, Ascorbic acid (400 µM) were used as positive control under the same assay conditions. Negative control was without any standard inhibitor or Hayanin. Lower absorbance at 517 nm represents higher DPPH scavenging activity. The percentage inhibition of DPPH radical of Hayanin was calculated.

2.4.2 Lipid Peroxidation inhibitory activity:

Spectrophotometric assay was done for evaluating antioxidant activity was based on the inhibition of peroxidation in Linoleic acid [33]. An assessment of oxidation was achieved by measurement of Thiobarbituric Acid Reactive Substances (TBARS) by fenton reaction [34]. 100 µL of Linoleic acid was subjected to peroxidation by 10:100 µmole of ferrous sulphate and ascorbic acid in final volume of 1 mL of Tris buffered saline (20 mM, pH 7.4, 150 mM NaCl). The reaction mixture was treated with or without Hayanin (10-50 µg), BHA (400 µM), Ascorbic acid (400 µM) and Curcumin (400 µM). The contents were incubated for 1 hour at 37 ^{0}C. The reaction was terminated by the addition of 10 µL of 5% phenol and 1 ml of 1% TCA. To this 1 mL of 1% TBA was added, the contents were kept in a boiling water bath for 15 min, cooled and centrifuged at 5000 g for 10 min. The absorbance of supernatants was measured at 535 nm.

2.4.3Hydroxyl Radical Scavenging activity:

The Hydroxyl Radical Scavenging activity of Hayanin was done according to the method of[35]with minor modifications. The reaction mixture containing $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_2 (1 mM) and 2-deoxy- D-ribose (2.8 mM) were mixed with and without Hayanin and the final reaction volume was made with potassium phosphate buffer (20 mM pH 7.4) to 1 mL and incubated for one hour at 37 ^{0}C. BHA, Curcumin and Ascorbic acid (400 µM) were used as standard antioxidants. To this add 1 ml of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA) and the mixture was heated at 95 ^{0}C in water bath for 15 min.Finally the reaction mixture was cooled on ice and centrifuged at 5000 g for 15 min. Absorbance of supernatant was measured at 532 nm using the negative control without any antioxidants was considered as 100% oxidation. The percentage of hydroxyl radical scavenging activity of Hayanin was determined.

2.4.4 Superoxide Radical Scavenging activity:

The Superoxide radical ($O_2^{\bullet-}$) scavenging activity was measured according to the method of[36], with minor modifications. The reaction mixture containing 100 µL of 30 mM EDTA (pH 7.4), 10 µL of 30 mM hypoxanthine in 50 mM NaOH, and 200 µL of 1.42 mM NBT with or without Hayanin and SOD was served as positive control at various concentrations ranging from 50-300 µg. The solution was pre-incubated at 37 ^{0}C for 3 min. and 100 µl of xanthine oxidase solution (0.5U/mL) was added to the mixture and incubated for one hour at 37 ^{0}C, and the volume was made upto 3 mL with 20 mM phosphate buffer (pH 7.4). The solution was incubated at room temperature for 20 minutes, and absorbance was measured at 560 nm. The control was kept without any inhibitor and the inhibitory effect of Hayanin on superoxide radical was calculated accordingly.

2.4.5 Reducing Potentiality:

The reducing power of Hayanin was determined according to the method of[37]. Aliquots of Hayanin (10-50 µg) and standard antioxidants were mixed with an equal volume of 0.2 M Phosphate buffer of pH 6.6 and 1% potassium ferricyanide. The mixture incubated at 50 ^{0}C for 20 min. An equal volume of 10% TCA added to the mixture and centrifuged at 3000 g for 10 min. The supernatant was mixed with distilled water and 0.1 % $FeCl_3$ at a ratio of 1:1:2 (v/v/v) and the absorbance were measured at 700 nm. The increased absorbance of the reaction mixture indicated increased reducing power.

2.5 Statistical analysis:

All experiments were carried out in triplicates to check the reproducibility of results. The data presented here are the averages of triplicate determinations and the standard deviations for all the values were <±5%.

3. RESULTS AND DISCUSSION

The proteinaceous protease inhibitor isolated form Horse gram seed coat was named as Hayanin [25]. In the present

study, the antioxidant potentiality of Hayanin was checked to know the effect of protease inhibitors on free radicals which are the main cause for many diseases. Hayanin with a molecular weight of ~26 kDa (SDS-PAGE) [25] had a potent protease inhibitory activity [25]. Upon isolating the Hayanin, it was subjected to the analysis of various components present at different stages of purification process. Table 1 shows that there is high concentration of protein as compared to the trace amounts of Sugars, Polyphenols, Flavonoids, Ascorbic acid and α-tocopherol, thereby determining that, protein is the main active principle.

Table 1: Proximate analysis of the protease inhibitor (Hayanin) at different stages of purification

Sample	Proteins	Total Sugars	Total Polyphenols	Flavonoids	Ascorbic acid	α-tocopherol
	mg %					
Crude Extract	2420	1685	2451	2129	1125	1359
$(NH_4)_2SO_4$fraction (65%)	1396	352	1593	725	59	671
Dialysed	808	98	0.224	0.152	0.051	0.032
Sephadex G-50 (HAYANIN)	9.829	0.002	0.003	0.001	-	-

(Values are represented as mean SD (n=3); mg % indicates mg per 100 g dry weight)

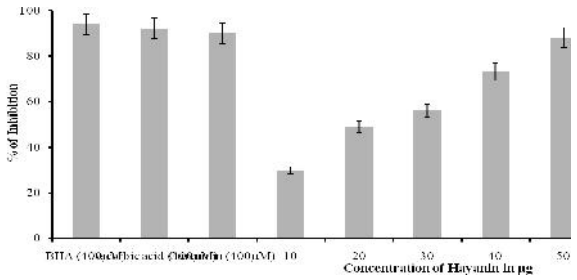

Fig 1: Dose dependent study of Hayanin on DPPH radical as compared with standard antioxidants, Bar indicates standard deviation from triplicate determination.

In DPPH assay, antioxidants were able to reduce the stable radical DPPH to diphenyl-picryl hydrazine- a yellow coloured compound. Figure 1 shows that the effect of Hayanin on DPPH radical has 88 % scavenging activity at 50µg concentration. The dose dependent study of Hayanin on DPPH radical shows a significant scavenging activity as compared to the standard antioxidants like BHA, Ascorbic acid and Curcumin (94, 92 and 90 % respectively) at 400 µM concentrations.

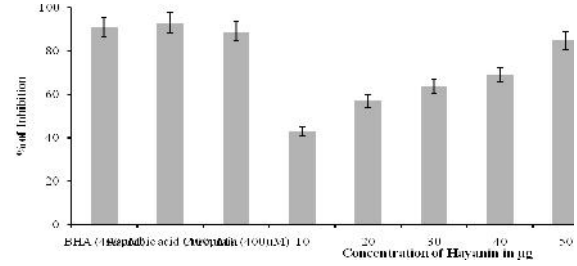

Fig 2: Dose dependent study of Hayanin on Lipid peroxidation assay which are compared with standard antioxidants, Bar indicates standard deviation from triplicate determination.

The lipid peroxidation inhibitory assay is determined by the production of malondialdehyde (MDA). The inhibitory effect of Hayanin against ferrous sulphate and ascorbic acid induced lipid peroxidation was estimated and Figure 2 shows that 85 % inhibition at 50 µg concentration as compared to the standard antioxidants like BHA, Ascorbic acid and Curcumin (91, 93 and 89 % respectively) at 400 µM concentrations.

The ability of Hayanin on Hydroxyl and Superoxide radical is shown in the Figure 3 and compared with the reference controls. Hydroxyl and Superoxide radical scavenging activity by Hayanin was found to be 77 % and 83% respectively at 50 µg concentration. Meanwhile BHA, Ascorbic acid and Curcumin displayed 86 & 84, 78 & 82 and 95 & 88 % scavenging potentiality, respectively at 400 µM concentration. Reducing power assay is one mechanism of action of antioxidants and may serve as an indicator of potential antioxidant activity. Figure 4 shows the reducing activity of Hayanin is indicated by the increased absorbance in a dose dependent manner as compared to the standard antioxidants.

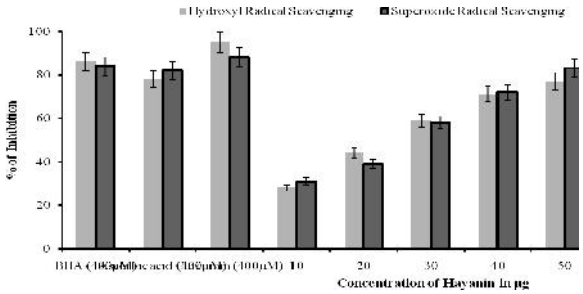

Fig 3: Dose dependent study of Hayanin on Hydroxyl and Superoxide radicals as compared with standard antioxidants, Bar indicates standard deviation from triplicate determinations.

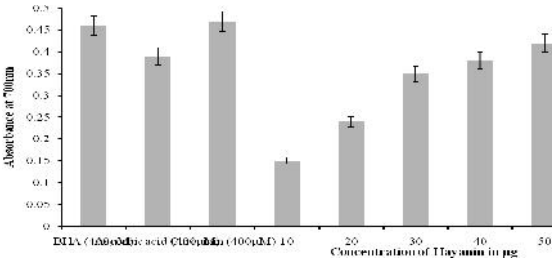

Fig 4: Dose dependent study of Hayanin as Reducing Power potentiality as compared with standard antioxidants, Bar indicates standard deviation from triplicate determinations.

A 33kDa trypsin inhibitor from sweet potato exhibited antioxidant activity against both DPPH and Hydroxyl radicals [38]. Low molecular weight (8 kDa) trypsin inhibitor from sweet potato leaves possessed the antioxidant activity against DPPH radical [39]. A 26 kDa protease inhibitor from the fruits of African nightshade had an effective antioxidant activity [1]. These suggest that a protein with a protease inhibitory activity had an effective antioxidant activity against different radicals.Protease inhibitors have tyrosine

residues with free hydroxyl group was reported to have antioxidant activities, which had an effect on DPPH, Hydroxyland Superoxide radical scavenging activity [40]. In the present study, the antioxidant activity of Hayanin may be due to the tyrosine residues. Hydroxyl radical is the most strongest among ROS, which reacts easily with amino acids, Proteins and DNA. The removal of hydroxyl radical is one of the most effective defences[1]. Superoxide radical produce a weak oxidants, but can produce more potent species like singlet oxygen which cause peroxidation of lipids. Hayanin effectively inhibits the superoxide radical activity thereby preventing the peroxidation of lipids.

4. CONCLUSION

The present study shows that the proteinaceous protease inhibitor- Hayanin from Horse gram seed coat exhibits effective antioxidant activities in different model systems. The Hayanin has potent antioxidant and free radical scavenging activities. As Hayanin is nontoxic, edible, easily available from plant source appears to be a promising candidate for development of an antioxidant protein. Further studies are needed to fully characterize and elucidate its mode of action.

5. ACKNOWLEDGEMENTS

We acknowledge the Sri Adichunchanagiri Mahasamsthana Math for financial support and PMSL gratefully acknowledge the fellowship by Rajiv Gandhi National Fellowship (UGC-RGNF), No. F. 14-2 (SC)/2010 (SA-III), Government of India, India.

6. REFERENCES

1. Meenu KVG, Murugan K. Antioxidant potentiality of partially purified protease inhibitor from the fruits of African nightshade (*Solanumaculeatissimum*Jacq.). W J Pharm PharmSci 2013; 2(6): 5166-5181.
2. Ranjith T, Kamalesh Y, Khim BK. Study of antioxidant, antibacterial and anti-inflammatory activity of cinnamon (*Cinamomumtamala*), ginger (*Zingiberofficinale*) and turmeric (*Curcuma longa*). Am J Li Sci 2013; 1(6): 273-277.
3. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 2010; 4(8):118-126.
4. Sandeep VM, Hemant K, Seong MK, Soo-Yeol S, Kippeum L, Dong-Kug C. Advances in Neuroprotective Ingredients of Medicinal Herbs by Using Cellular and Animal Models of Parkinson's Disease. Evid Based Complement Alternat Med 2013; 2013: 957875.
5. Ningappa MB, Srinivas L. Purification and characterization of approximately 35 kDa antioxidant protein from curry leaves (Murrayakoenigii L.). Toxicol in Vitro 2008; 22: 699–709.
6. Lai HY, Kim KH. Blechnumorientale Linn - a fern with potential as antioxidant, anticancer and antibacterial agent. BMC Complement Altern Med 2010; 10: 15-22.
7. Ames BN. Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative diseases. Science 1983; 221: 1256-1264.
8. Gey KF. The antioxidant hypothesis of cardiovascular disease: epidemiology and mechanisms. BiochemSocTrans 1990; 18: 1041-1045.
9. Smith MA, Perry G, Richey PL, Sayre LM, Anderson VE, Beal MF, Kowal N. Oxidative damage in Alzheimer's. Nature 1996;382: 120-121.
10. Diaz MN, Frei B, Vita JA, Keaney JF. Antioxidants and atherosclerotic heart disease. N. Engl J Med 1997; 337: 408-416.
11. B. Halliwell and J. Gutteridge.Free Radicals in Biology and Medicine, Clarendon Press: New York, USA, 1999; 20–37.
12. Sohal RS, Mockett RJ, Orr WC. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Free RadicBiol Med 2002; 33: 575–586.
13. Chowienczyk PJ, Brett SE, Gopaul NK, Meeking D, Marchetti M, Russell-Jones DL,Anggard E E, Ritter JM. Oral treatment with an antioxidant (raxofelast) reduces oxidativestress and improves endothelial function in men with type II diabetes. Diabetologia2000;43:974–977.
14. Parthasarathy S, Santanam N, Ramachandran S, Meilhac O. Potential role of oxidized lipids and lipoproteins in antioxidant defense. Free Radic Res 2000;33: 197–215.
15. DeNicola GM, Karreth FA, Humpton TJ, Gopinathan A, Wei C, Frese K, Mangal D, Yu KH, Yeo CJ, Calhoun ES,et al.,. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. Nature 2011; 475: 106–109.
16. Haenen GR, Bast A. The use of vitamin A supplements in self- medication. Therapie 2002; 57: 119–122.
17. Watts P. BHA isomers and hyperplasia. Food ChemToxicol 1975; 23: 639–640.
18. Srinivas L, Shalini VK. DNA damage by smoke: protection by turmeric and other inhibitors of ROS. Free Rad Biol Med 1991; 11: 277–283.
19. Srinivas L, Shalini VK,Shylaja M. Turmerin:a water-soluble antioxidant peptide from turmeric(Curcuma longa).Arch Biochem Biophys 1992; 292: 617–623.
20. Ryan CA. Protease inhibitor gene families: Strategies for transformation to improve plant defenses against herbivores. BioEssays1989;10: 20-24.
21. Benjakul S, Visessanguan W, et al., Isolation and characterization of trypsin inhibitors from some Thai legume seeds. J Food Biochem2000; 24: 107-127.
22. M. Richardson , Seed storage proteins: The Enzyme inhibitors. In Methods in Plant Biochemistry: Amino Acids, proteins and Nucleic Acids, Vol 5, Rogers LJ (ed), Academic Press: New York, 1991; 259-305.

23. BibhabasuHazra, RhitajitSarkar, et al., Studies on antioxidant and antiradical activities of Dolichosbiflorus seed extract, Afr J Biotechnol 2009; 8(16): 3927-3933.
24. Yadahally NS, Vadakkoot BS, Vishwas MP. Variability in the Distribution of Phenolic Compounds in Milled Fractions of Chickpea and Horse Gram: Evaluation of Their Antioxidant Properties. J Agric Food Chem 2010; 58 (14): 8322–8330.
25. Prabhu MSL, Leela S. Isolation, purification and characterization of a protease inhibitor-Hayanin from Horse gram [Macrotylomauniflorum (Lam.) Verdc.] seed coat. Int J Pharm Phytopharmacol Res 2015; 5(2): 1-8.
26. Bradford MM, A rapid and sensitive method for the quantification of microgram of protein utilizing the principle of protein dye binding. Anal Biochem 1976; 72: 248-254.
27. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956; 28: 350.
28. Kujala TS, Loponen JM, Klika KD, Pihlaja K. Phenolics andbetacyanins in red beetroot (Beta vulgaris) root: Distribution and effect of cold storage on the content of total phenolics and three individual compounds. J Agri Food Chem 2000; 48: 5338-5342.
29. Woisky R, Salatino A. Analysis of Propolis: some parameters and procedures for chemical quality control. J Apic Res 1998; 37: 99-105.
30. S. Sadasivam and A. Manickam.Identification of Proteins, Biochemical Methods.Second edition, New Age International Publishers, 1997, 33.
31. AOAC. Official methods and recommended practices of American Oil Chemist Society.Sixth edition, American Oil Chemist Society Press, California, USA, 2013.
32. Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of Xanthan on the auto oxidation of soybean oil in cyclodextrin emulsion. J Agri Food Chem 1992; 40: 945-948.
33. Shimazaki H, Ueta N, Mowri HO, Inoue K. Formation of age pigment like fluorescent substances during peroxidation of lipids in model membranes. Biochem Biophys Acta 1984; 792(2): 123-129.
34. Fenton HJH. Oxidation of tartaric acid in the presence of iron. J ChemSoc 1984; 106: 899-910.
35. Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: a simple assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem 1987; 165: 215-219.
36. Lee J, Kwon ES, Kim DW, Cha J, Roe JH. Regulation and the role of Cu, Zn-containing superoxide dismutase in cell cycle progression of Schizo saccharo mycespombe. Biochem Biophys Res Commun 2002; 297: 854–862.
37. Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J Agri Food Chem 1995; 43: 27–32.
38. Wen CH, Yen CC, Hsien JC, Yaw HL, Ling LY, Mei HL. Antioxidant activities of trypsin Inhibitor, a 33kDa root storage protein of sweet potato (Ipomoea batatas (L.) Lam cv. Tainong 57). J Agri Food Chem 2001; 49: 2978-2981.
39. Guan JH, Ying CL, Jeng SD, Hsien JC, Jung CL, Shyh SH, Shu JC, Yaw HL. A novel trypsin inhibitor from sweet potato (Ipomoea batatas Lam.) leaves and its synthesized peptides with antioxidant activities in vitro. Botanical Studies 2012; 53: 215-222.
40. Glucin I. Comparison of invitro antioxidant and antiradical avitvities of L-tyrosine and L- Dopa. Amino acids 2007; 32: 431-438.

Conflict of Interest: None