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RESEARCH ARTICLE

ANTIOXIDANT ACTIVITIES OF PIPPALI (*PIPER LONGUM*) PROTEINS

DINESHA R[1], CHIKKANNA D[2*]
E-mail: dr.chikkanna@gmail

1. Adichunchanagiri Biotechnology and Cancer Research Institute, B.G. Nagara
2. Adichunchanagiri Institute of Medical Sciences, B.G. Nagara

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Abstract:
The study was conducted to find and evaluate antioxidant activities of proteins isolated from boiling water extract of Pippali (*Piper longum)* (long pepper). The antioxidant activity of the proteins was analyzed using Hydroxyl radical scavenging assay and lipid peroxidation inhibition assays. The proteins were analyzed for their thermal stability. The cytotoxic studies showed that, the proteins are non toxic. The results obtained are promising when compared with standard antioxidants Vitamin E, BHA, and Ascorbic acid.

Keywords: Antioxidant, Stability, Pippali, Proteins, Cytotoxic

Introduction

In ancient times, spices, spice extracts, and herbs have been thought to cure diseases. Herbs and spices have been used to enhance flavors and fragrances of food, as well as for their medicinal purposes. Many spices have antioxidant properties and also prevent food rancidity. (Lai, 2004). It is reported that, volatile oils of few spices have been found to have antibacterial activity against many food-borne pathogens. (Dorman and Deans, 2000). It is reported that, curcumin from Turmeric, reduced beta-amyloid and plaque burden in the brain, increasing cognitive function in elderly patients (Ng et al., 2006).

The Pippali (*Piper longum*) or Long pepper belongs to *Piperaceae* family have strong aromatic and medicinal properties. It is commonly known as ginger, and exist roughly 1,920 currently accepted species in 13 genera (Kirtikar, and Basu, 1933).

The vast majority of peppers can be found within the two main genera: *Piper* (2000 species) and *Peperomia* (1600 species) widely used in North-eastern and Southern India and Sri Lanka. *Piper longum* is widely used in Siddha, Ayurveda and Unani systems of medicine, particularly for diseases of the respiratory tract (Williamsons, 2002, Maitreyi Zaveri et al, 2010).

The present study reveals the antioxidant properties of boiling water extract of Long pepper (*Piper longum*) proteins and its non cytotoxic nature. The boiling water extract of the above still retained its antioxidant capacity.

Materials and methods:
Long pepper (*Piper longum*) was procured from authenticate source. BHA, α-tocopherol, 2-deoxy ribose was purchased from Sigma (St. Louis, USA). Other chemicals unless otherwise mentioned were procured from Merck.

Isolation of protein from boiling water extract of Long pepper (*Piper longum*)
The procured Long pepper was cleaned and finely powdered (100 mesh British Pharmacopia) stored. Five grams of powder mixed with boiling double distilled water, vortexed for four hours at 200 rpm, centrifuged for 20 minutes at 10000 rpm at 4°C, and the supernatant was filtered and the supernatant brought to 65% saturation with ammonium sulphate. Further the ammonium sulphate precipitate was dialyzed against water using 2kDa dialysis membrane for 72 hours with a regular interval of 6 hours.

Proximate analysis:
The proximate analysis such as protein, total sugars, polyphenols, flavonoids, ascorbic acid, α-tocopherol was done.

Estimation of Protein
The protein content of the both extracts was estimated according to Bradford's method (Bradford, 1976) using bovine serum albumin as standard. Aliquots were made up to 0.1ml with distilled water and 0.9 ml of Bradford's reagent was added. The total protein content was calculated using the standard curve.

Estimation of total sugar
The total sugar concentration Long pepper extract was estimated by Dubois method (Dubois et al., 1956) and Dextrose used as standard. Different aliquots were made up to 1ml with distilled water followed by the addition of 1ml of 5% phenol and 5ml of concentrated sulphuric acid and was read at 520nm. The sugar concentration was calculated accordingly.

Estimation of total phenolic
Total content of phenolic was determined by the method of Folin-ciocalteau reaction using Gallic acid as standard (Kujala et al., 2000). The extract and the standard Gallic acid were dissolved in 0.5ml of water and were mixed with 500µl of 50% Folin- ciocalteau reagent. The mixture was then allowed to stand for 10min followed by the addition of 1.0ml of 20% Sodium carbonate. Incubated at 37^0C, 10 min and the absorbance of the supernatant were read at 730nm. The total phenolics content was expressed as Gallic acid equivalents in milligrams per gram of powder.

Estimation of flavonoids:
Total flavonoid content was determined colorimetrically using spectrophotometer (Woisky and Salatino, 1998). Standard calibration curve was prepared using Quercetin. 10mg of Quercetin was dissolved in 80% ethanol and then diluted solutions were separately mixed with 1.5ml of 95% ethanol, 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30min, the absorbance of the reaction mixture was measured at 415nm by using proper controls. The total flavonoid content was calculated accordingly.

Antioxidant activity:
Antioxidant activity of the protein rich long pepper extract was studied in different model system to study their antioxidant activities.

Lipid peroxidation inhibition activity:
A simple spectrophotometric assay was done for evaluating antioxidant activity was based on the inhibition of peroxidation in Linoleic acid (Shimazaki et al., 1984). An assessment of oxidation was achieved by measurement of thiobarbituric acid reactive substances (Dahle et al., 1962). 100 µ1 of Linoleic acid was subjected to peroxidation by 10:100 µmol of ferrous sulphate and ascorbic acid (Fenton, 1984) 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 long pepper protein (25µg), BHA (400uM), α-tocopherol (400uM) and Curcumin (400uM). The contents were incubated for 1 hour at 37°C. The reaction was terminated by the addition of 10 µl of 5% phenol and 1 ml of 1% trichloroacetic acid (TCA). To each system 1 ml of 1% thiobarbituric acid (TBA) was added, the contents were kept in a boiling water bath for 15 min, cooled and centrifuged at 6000 rpm for 10 min. The absorbance of supernatants was measured colorimetrically at 535 nm. Appropriate blanks were included for each measurement. The negative control without any test sample was considered as 100% peroxidation. The control was without any antioxidant or test sample (Table-2).

Hydroxyl radical scavenging activity of extracts:
The hydroxyl radical scavenging activity of Long pepper protein was done (Halliwell et al., 1987). 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 or without Long pepper protein (25µg) in 1 ml final reaction volume made with potassium phosphate buffer (20 mM pH 7.4) and incubated for one hour at 37°C. BHA and Curcumin (400µM) were used as positive control. The mixture was heated at 95°C in water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M

NaOH containing 0.02% BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm using the negative control without any antioxidant was considered 100% oxidation. The percentage hydroxyl radical scavenging activity of protein was determined.

Thermal stability studies:
The extracts were kept in boiling water bath for 60 minutes and later the antioxidant capacity of the extract was analyzed using Hydroxyl radical scavenging activity.

Cytotoxicity assay
Cytotoxicity was determined according to the method of Chwetzoff, Tsunasawa, Sakiyama, and Ménez (1989). EAT cells were suspended in Tyrode's solution (5 x 10^6 cells/2 ml) and incubated with Long pepper protein (0–250 µg) for 30 min. Tryphan blue saline (1%, 100 µl) solution was then added, and unstained (viable) cells were counted using a haemocytometer. The percentage of viable cells was determined by comparing with the number of viable cells in the control (designated as 100%).

Statistical analysis
Statistical analysis was done using SPSS (Windows version 10.0.1; SPSS Inc., Chicago, IL) using a one-way student's t-test; $p < 0.05$ was considered as statistically significant, when comparing with relevant controls. All results refer to mean ± SD.

Results and discussion
Earlier it was reported that, a 35kDa protein from Curry leaves showed both antioxidant (Ningappa et al., 2008). It was reported that, a 15kDa protein from Sundakai (*Solanum torvum*) (Sivapriya and Leela Srinivas 2007) and a 28 kDa glyco protein from Turmeric boiling water extract (Dinesha and Leela Srinivas, 2011) were reported for their antioxidant activities and effectiveness of boiling water extract of Star anise against free radicals induced DNA damage (Dinesha et al., 2014). The above information and results encouraged us to systematically study the different proteins from herbs and spice sources for their effective antioxidant activities. For the crude protein extract obtained using Ammonium sulphate precipitation, the proximate analysis results showed that the extract contains negligible amount of polyphenols, flavonoids, free sugars and rich with proteins. The antioxidant activity of the above proteins rich extract was analyzed with different model systems like hydroxyl radical scavenging capacity and lipid peroxide inhibitory activity. As explained in the materials and methods, the protein was isolated from the extract using ammonium sulphate precipitation method followed with dialysis against water to remove unwanted salt contents. Further, the protein rich extract was analyzed by proximately analysis to confirm the extract rich with only proteins. As shown in the table -1, the extract rich with proteins and contains negligible amount of sugars.

Further the extract was subjected to analyze for its antioxidant ability against Hydroxyl radical scavenging activity and inhibition of formation of lipid peroxidation as in the Table-2. Here, Alpha tocopherol, BHA and Ascorbic acid are used as standard antioxidants at 400µM concentration and 10µg of Long Pepper protein extract was used. When compared to the standards, Long pepper proteins showed an inhibitory activity of 81% and 83% and the results are very promising. Further the extract and Ascorbic acid were subjected to thermal stability test by keeping the extract in boiling water bath for 60 minutes and further its antioxidant activity was analyzed using Hydroxyl radical scavenging activity. It showed 65% of inhibition when compared to Ascorbic acid which showed only 41%, which confirms that, the Long pepper proteins are more thermal stable.

Further we investigated the toxicity of proteins of Long pepper using human erythrocyte cells. It was observed that, there was no decrease in the number of viable cells when Long pepper proteins were added. These results clearly indicate that proteins of Long pepper is non-toxic for cells.

In conclusion, the obtained results of proteins of Long pepper are very promising and interesting and hence further studies about its medicinal properties need to be done.

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Reference:

1. Bradford M.M., 1976, A rapid and sensitive method for the quantification of microgram of protein utilizing the principle dye binding. Annual biochem. 72: 245.

2. Chwetzoff S., Tsunasawa S., Sakiyama F., Ménez A Nigexine., 1989, A phospholipase A2 from cobra venom with cytotoxic properties not related to esterase activity. Purification, amino acid sequence, and biological properties, The Journal of biological chemistry 264 , 13289-13297

Table-1 : Proximately analysis of protein rich Long pepper extract

Tests	Dialyzed extract (g%)
Protein	0.812
Sugars	0.002
Polyphenols	Nil
Flavonoids	Nil

The results are mean ± SD (n=6)

Table-2: Antioxidant activities of proteins rich Long pepper extract

Sl. No.	Antioxidants	Concentration	% of inhibition	
			Hydroxyl radicals	Lipid peroxidation
01	**α-tocopherol**	**400µM**	**85**	**92**
02	**BHA**	**400µM**	**74**	**79**
03	**Ascorbic acid**	**400µM**	**78**	**64**
04	**Protein extract of Long pepper**	**10µg**	**81**	**83**

The results are mean ± SD (n=6)

3. Dahle L.K., Hill E.G., Holman R.T., 1962, The thiobarbituric acid reaction and the autooxidants of polyunsaturated fatty acid methyl esters. Archives of Biochemistry and Biophysics 98, 253–261.

4. Dinesha Ramadas and Leela Srinivas, 2011, Antioxidant effects of 28kDa protein from turmeric (*Curcuma longa L*), *Asian J. Pharm. Clin. Res*., **4**, Suppl 1, 75-79.
5. Dinesha R., Thammannagowda S.S., Shwetha K.L., Prabhu M.S.L., Madhu C.S., Leela Srinivas, 2014, the antioxidant and DNA protectant activities of Star Anise (*Illicium verum*) aqueous extracts, Journal of Pharmacognosy and Phytochemistry; 2 (5): 98-103.
6. Dorman, H.J., Deans, S.G. Antimicrobial Agents from Plants: Antibacterial Activity of Plant Volatile Oils. 2000, J. Appl. Microbiology, 88, 308-16.
7. Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F.. 1956, Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28: 350.

8. Halliwell B., Gutteridge J.M.C., Aruoma O.I.. 1987, The deoxyribose method: a simple assay for determination of rate constants for reactions of hydroxyl radicals, Anal. Biochem. 165; 215–219.

9. Kirtikar K.R., Basu B.D., Indian Medicinal Plants, 1933, 2nd Edn, Lalit Mohan Basu Publications, Allahabad, pp.2131-2133.

10. Kujala T.S., Loponen J.M., Klika K.D., Pihlaja K., 2000, Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root Distribution and effect of cold storage on the content of total phenolics and three individual compounds. Journal of Agricultural and Food Chemistry, 48, 5338–5342.

11. Lai P.K., Roy J., 2004, Antimicrobial and Chemopreventative Properties of Herbs and Spices, Current Medical Chemistry; 11 (11): 1451-1460.
12. Maitreyi Zaveri, Amit Khandhar, Samir Patel, Archita Patel, 2010, Chemistry and pharmacology of Piper longum l., International Journal of Pharmaceutical Sciences Review and Research, Vol 5-1, 010: 67-76.
13. Ng, Tze-Pin, Peak-Chiang Chiam, Theresa Lee, Hong-Choon Chua, Leslie Lim, Ee-Heok Kua. 2006, Curry Consumption and Cognitive Function in the Elderly, Am *J* Epidemiol;164(9):898-906.
14. Ningappa M.B., Dinesha R., Leela Srinivas, 2008, Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (*Murraya koenigii L.*) extracts. Food Chemistry, 106 :720–728.
15. Parmar N.S., Jain S.C., Bisht K.S., Jain R., Taneja P., Jha A., Tyagi O.D., Prasad A.K., Wengel J., Olsen C.E., Boll P.M., 1997, Oxygen deprivation stress in a changing environment, *Phytochem,* , 46(4), 597-599.
16. Sivapriya M., Srinivas L., 2007, Isolation and purification of a novel antioxidant protein from the water extract of sundakai (*Solanum torvum*) seeds. Food Chem; 104; 510–517.
17. Williamsons, 2002, Piper longum, Major herbs of Ayurveda, 1st Edn, Dabur research foundation, Dabur Ayurvet Limited, Elsevier health science, pp. 225-227.
18. Woisky R., Salatino A., 1998, Analysis of Propolis: some parameters and procedures for chemical quality control. J. Apic. Res. 37: 99-105.