

Evaluation of Antioxidant and Antidiabetic Potential of Crude Protein Extract of Pumpkin Seeds (*Cucurbita maxima* L.)

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Submission Date: 22-04-2020; Revision Date: 26-06-2020; Accepted Date: 22-07-2020

ABSTRACT

Pumpkin seeds are a rich source of protein and fibre. Proteins present in pumpkin seeds have received less attention in spite of their significant biological activities. The objective of the present study was to evaluate the antioxidant and antidiabetic activity of crude protein obtained from pumpkin seeds. Protein was extracted using pre-chilled phosphate buffer extraction (0.2M; pH-7), followed by acetone precipitation and dialysis. The protein content of pumpkin seeds was estimated using Bradford's method. The electrophoretic profile was determined using SDS PAGE. Antioxidant activity was determined using DPPH[•] radical, ABTS^{•+} radical cation, Fe³⁺ reduction and phosphomolybdenum reduction assay. Antidiabetic activity was determined using alpha amylase inhibition assay. Mean values of different concentrations were tested for significance using one way Anova followed by Tukey's test. The protein content of pumpkin seed was 100.48 ± 0.72 mg/mL and the molecular weight of the extracted proteins ranged from 25 to 45 kDa. The proteins present in pumpkin seeds showed significant antioxidant activities in a dose dependent manner ($p < 0.05$). The IC₅₀ value of DPPH[•] radical and ABTS^{•+} radical scavenging assay was 670 µg/mL and 3.49 µg/mL. Pumpkin seed proteins showed significant alpha-amylase inhibitory activity ($p < 0.05$) and the IC₅₀ value was 127.94 µg/mL. The results of the present study indicate the biological health promoting effects of plant proteins and their use as a functional food component.

Key words: Antidiabetic activity, Antioxidant potential, Pumpkin seeds, Plant protein, SDS-PAGE.

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INTRODUCTION

Cucurbits are one of the largest and diverse groups of plant families that are cultivated, because the seeds and fruit of these plants exhibit wide spectrum of biological activities. Pumpkin is one of the most important creeping plant crops of cucurbitaceae family that is widely cultivated in both tropical and sub-tropical countries. Economically, there are three important *Cucurbita* species

namely *Cucurbita pepo*, *Cucurbita maxima* and *Cucurbita moschata* that are widely distributed in agricultural regions. Pumpkin has been used as a medicinal agent for curing several ailments in America, Argentina, Brazil, China, India and Mexico.^[1] Phytochemicals, polysaccharides, sterols, proteins and peptides present in pumpkin provide a wide array of therapeutic health benefits. Some of the reported health benefits of pumpkin include antidiabetic, anticancer, anti-inflammatory, antihypertensive, antimicrobial, antioxidant potential and preventing kidney stone formation.^[2] Pumpkin is also famous for its low fat protein rich seeds that are consumed in raw or roasted form. These seeds are rich in essential micronutrients, proteins, polyunsaturated fatty acids and phytochemicals.^[3,4] The Chinese and Ayurvedic medicinal system has utilized pumpkin

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DOI :
10.5530/ajbls.2020.9.23

seeds as a vermifuge, as a diuretic agent to treat kidney disorders, to alleviate prostate diseases and for curing skin infections.^[5] Foods rich in protein contain bioactive peptides which possess physiological functions beyond their basic roles of providing nutritional benefits.^[6] Bioactive peptides are specific protein fragments that have a positive impact on body functions. Most of the biological activities of these peptides are encrypted within the primary sequence of the native protein that are released by enzymatic hydrolysis, proteolysis or by food processing.^[7] Peptides and peptide fractions isolated from various food sources are reported to have bioactive function such as immunomodulatory, hypocholesterolemic, antihypertensive, antioxidative, anticancer, antimicrobial and mineral binding properties.^[8] Antioxidants protect the body from oxidative stress by neutralizing the harmful effects of free radicals. Oxidative stress is involved in the development and progression of complications associated with type 2 diabetes mellitus. In hyperglycaemic conditions, continuous production of free radicals along with changes in the functions of enzymatic antioxidants occur in the body. Thus natural products functioning as both antioxidant and antidiabetic agents are useful in the treatment of diabetes mellitus. Use of plant proteins and protein hydrolysates as antioxidant and antidiabetic components are gaining interest as they are chief components of the redox homeostasis maintenance system in cell metabolism.^[9] Pumpkin seeds are a rich source of protein that can be effectively utilized as a functional food component. Therefore, the objective of the present study was to extract the proteins present in pumpkin seeds and evaluate its antioxidant and antidiabetic potential.

MATERIALS AND METHODS

Extraction of pumpkin seed protein: Proteins present in pumpkin seeds (*Curcubita maxima* L.) was extracted using the method described by Terras *et al.*^[10] and Osborn *et al.*^[11] with slight modifications. One hundred grams (100g) of pumpkin seeds were powdered and mixed with 100mL of pre-chilled phosphate buffer solution (0.2M; pH- 7). This solution was filtered and centrifuged at 7000 rpm for 20 min. The resulting supernatant was collected. This supernatant was mixed with 65% ice cold acetone at regular intervals and was subjected to constant mixing using a magnetic stirrer for 20 min. Following this, the solution was again centrifuged at 10,000 rpm for 15 min and finally the supernatant was discarded. The resulting pellet was collected and dissolved in minimal amount of sterile distilled water for

performing dialysis. Dialysis was carried out to remove all unwanted molecules using a pre-treated dialysis membrane. After dialysis, the partially purified protein sample was collected and stored at 4°C until further use. Protein content was estimated using Bradford's method. Bovine serum albumin was used as standard reference.

Determination of molecular weight using SDS

PAGE: The molecular weight of the partially purified protein was determined using SDS PAGE following the method described by Laemmli.^[12] In the present study, gradient mini gels (resolving gel pH-8.8, stacking gel buffer pH-6.8 and tank buffer pH-8.3) were used to separate the proteins according to their molecular weight. The test sample was dissolved in sample buffer. The sample buffer consisted of Tris – buffer, 4% SDS, glycerol and a tracking dye bromophenol blue (BPB). Sample was mixed with 10µL of sample buffer and denatured by heating at 100°C for three minutes. Test sample and the standard protein ladder were loaded into the wells carefully using a micropipette. Care was taken to make sure that the sample and the standard settled down at the bottom of the well evenly before running of the gel. The gel was run for 3 hr at 50-60V at room temperature until the dye reached the bottom of the gel. Following this, the gel was removed carefully and was stained for colour development. Staining solution consisted of 0.1 g (w/v) of Coomassie Brilliant Blue R-250 (CBB) along with methanol, acetic acid and distilled water (25:6:19 v/v). The gel was stained for 3-4 hr with constant agitation in a gel rocker at a desired speed. Following this, the gel was subjected to destaining process and was monitored till bands were observed. Destaining solution consisted of methanol: acetic acid: distilled water (50:10:40 v/v).

Antioxidant assays

DPPH• radical scavenging assay: The antioxidant activity of pumpkin seed protein was measured on the basis of scavenging activity of stable free radical DPPH•.^[13] Different concentrations of the sample (200 – 1000 µg/mL) were mixed with 0.1mM of DPPH solution in methanol. The setup was then allowed to stand at room temperature for 30 min in a dark place. Absorbance was read at 517 nm using an UV vis spectrophotometer.

ABTS•+ radical scavenging assay: ABTS•+ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in dark at room temperature for a period of 12-16 hr before use. The ABTS solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH-7.4) to an absorbance of 0.70±0.02 at 734 nm. Different concentrations of the sample (2-10µg/mL)

were mixed with 1 mL of diluted ABTS^{•+} solution and incubated for 10 min. The absorbance was measured spectrophotometrically at 734 nm.^[14]

Fe³⁺ reduction assay: The Fe³⁺ reducing capacity was done according to the method described by Yen and Chen.^[15] Different concentrations of the sample (20-120µg/mL) were mixed with 1 mL of phosphate buffer (0.2 M, pH- 6.6) and 1 mL of potassium ferricyanide (1% w/v). The mixture was incubated at 50°C for 20 min and 500 µL of 10% trichloroacetic acid (w/v) was added to the mixture. Then 0.1 mL of 0.1% of freshly prepared ferric chloride was added and the absorbance was measured at 700 nm using an UV vis spectrophotometer.

Phosphomolybdenum reduction assay: Phosphomolybdenum reduction assay is based on the formation of green phosphomolybdenum complex.^[16] Various concentrations of the sample (20-120µg/mL) were combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. The samples were cooled to room temperature and the absorbance was measured spectrophotometrically at 695 nm.

In vitro antidiabetic assay

Alpha amylase inhibition assay: Alpha amylase inhibition assay was carried out according to the method described by Kotowaroo *et al.*^[17] This assay is based on starch-iodine test. Different concentrations of the sample (20-120µg/mL) were added to 500 µL of 0.02 M sodium phosphate buffer (pH- 6.9 containing 6 mM sodium chloride). Ten microliters (10µL) of alpha amylase solution was added and incubated for 10 min at room temperature. Then 500 µL of soluble starch (1%, w/v) was added and incubated for 1 hr. One hundred microliters (100 µL) of 1N dilute hydrochloric acid was

added to stop the enzymatic reaction, followed by the addition of 200 µL of freshly prepared iodine solution. The colour change was noted and the absorbance was read at 565 nm using an UV visible spectrophotometer.

Statistical analysis: Data analysis was done using SPSS software version 15.0. Values are presented as mean ± standard deviation of triplicates. Mean values of different concentrations were tested for significance using one way Anova followed by Tukey's test. IC₅₀ value was calculated using graph pad prism software.

RESULTS

Upon completion of gel running, the gel was rinsed with sterile distilled water to remove buffer salts used for electrophoresis which was followed by gel staining. Coomassie Brilliant Blue R-250 (CBB) binds with proteins through ionic and Van Der Waals interactions. The protein content was found to be 100.48 ± 0.72 mg/mL. In the present study, the degradation pattern of crude protein extracted from pumpkin seeds was determined using SDS-PAGE. Figure 1 shows the electrophoretic profile of pumpkin seed protein. Three distinct bands were observed after complete destaining process and the molecular weight ranged between 25 and 45 kDa.

Antioxidant activity: Free radicals are highly reactive chemical species that cause damage to bio molecules such as lipids, proteins and nucleic acids. DPPH[•] is a stable nitrogen centered free radical that can readily accept a proton. DPPH[•] is dark purple in colour and the absorbance can be measured at 517 nm. In the presence of antioxidant compounds that have a free proton to donate, DPPH gets scavenged which is evident through colour change from purple to yellow along with subsequent reduction in absorbance.^[18] ABTS^{•+} is a green blue cationic radical that is produced by the reaction between ABTS^{•+} and potassium persulphate. In the presence of an antioxidant molecule, decolourization of ABTS^{•+} radical takes place that can be measured spectrophotometrically at 734nm. Greater the decolourization, greater is the antioxidant activity.^[19] In the present study, results of free radical scavenging activities are expressed in terms of percentage inhibition of free radicals (Table 1). Results indicate that the ability to scavenge free radicals significantly increased with increase in concentration ($p < 0.05$). Antioxidants neutralize the harmful effect of free radicals through mechanisms such as binding of transition metal ion catalyst, preventing chain initiation reactions and reducing capacity/power.^[20] In the present study, the reducing power of pumpkin seed protein was evaluated using Fe³⁺

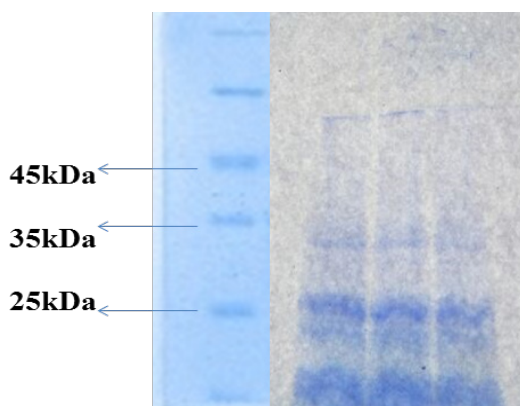


Figure 1: Electrophoretic profile of pumpkin seed protein.

Table 1: Free radical scavenging activity of pumpkin seed protein.

| DPPH [•] radical scavenging assay | | ABTS ^{•+} radical scavenging assay | |
|--|-------------------------------|---|-------------------------------|
| Concentration (µg/mL) | % inhibition of free radicals | Concentration (µg/mL) | % inhibition of free radicals |
| 200 | 24.17 ± 0.86 ^a | 2 | 41.08 ± 0.36 ^a |
| 400 | 33.02 ± 3.86 ^a | 4 | 57.40 ± 0.36 ^b |
| 600 | 45.57 ± 4.61 ^b | 6 | 61.73 ± 0.72 ^c |
| 800 | 59.73 ± 0.87 ^c | 8 | 67.35 ± 0.72 ^d |
| 1000 | 61.84 ± 1.10 ^c | 10 | 80.87 ± 0.36 ^e |

Values are the mean of triplicates

Different superscripts in a column are significantly different ($p < 0.05$)

reduction and phosphomolybdenum reduction assays. In Fe³⁺ reduction method, the ability of pumpkin seed protein to reduce Fe³⁺ ferric cyanide complex to Fe²⁺ ferrous cyanide complex was determined. On the other hand, phosphomolybdenum reduction assay is based on reduction of phosphomolybdenum (VI) is reduced to phosphomolybdate (V). Higher the absorbance, greater is the reducing power. Results of reducing power assays indicate that the reducing power of pumpkin seed protein increased linearly with increase in concentration (Table 2).

Table 2: Fe³⁺ reduction and Phosphomolybdenum reduction activity of pumpkin seed protein.

| Fe ³⁺ reducing assay | | | Phosphomolybdenum reduction assay | | |
|---------------------------------|---------------------------|---------------------------|-----------------------------------|--------------------------|----------------------------|
| Concentration (µg/mL) | Absorbance at 700 nm | | Concentration (µg/mL) | Absorbance at 695 nm | |
| | Pumpkin seed protein | Ascorbic acid | | Pumpkin seed protein | Ascorbic acid |
| 20 | 0.12 ± 0.01 ^a | 0.20 ± 0.10 ^a | 20 | 0.52 ± 0.06 ^a | 0.76 ± 0.02 ^a |
| 40 | 0.30 ± 0.03 ^{ab} | 0.34 ± 0.03 ^{ab} | 40 | 0.70 ± 0.03 ^b | 0.80 ± 0.01 ^{ab} |
| 60 | 0.44 ± 0.05 ^{ab} | 0.40 ± 0.12 ^{ab} | 60 | 0.80 ± 0.04 ^c | 0.82 ± 0.02 ^{abc} |
| 80 | 0.46 ± 0.04 ^{ab} | 0.47 ± 0.09 ^{bc} | 80 | 0.85 ± 0.04 ^d | 0.86 ± 0.02 ^{bc} |
| 100 | 0.55 ± 0.14 ^b | 0.52 ± 0.07 ^c | 100 | 0.86 ± 0.05 ^d | 0.86 ± 0.02 ^{bc} |
| 120 | 0.58 ± 0.15 ^b | 0.62 ± 0.02 ^c | 120 | 0.89 ± 0.04 ^d | 0.91 ± 0.03 ^c |

Values are the mean of triplicates

Mean values having the same superscript do not vary significantly at $p < 0.05$

Antidiabetic activity: Alpha amylase is the first enteric enzyme that initializes the hydrolysis of complex carbohydrates into mixture of oligosaccharides and disaccharides. Inhibiting the action of alpha amylase reduces the risk of post prandial hyperglycemia.^[21] The ability of pumpkin seed protein to inhibit the action of alpha amylase significantly increased in a dose dependent manner ($p < 0.05$) (Table 3).

DISCUSSION

Currently, different therapies are available for treating hyperglycaemia. One among them is the usage of pharmacological drugs. However, over usage of pharmacological drugs are associated with side effects. Food derived proteins and peptides possess beneficial physiological functions such as antioxidant, anticancer, antidiabetic, antihypertensive and anti-inflammatory functions. Plant foods rich in proteins can be used as a remedy for controlling high blood sugar levels.^[22] Oilseeds are a rich source of proteins, amino acids, unsaturated fatty acids, dietary fibre, vitamins and minerals. Pumpkin seeds can be effectively utilized as

a functional food component due to its high protein content and unique amino acid composition. The present study aimed at extracting the proteins present in pumpkin seeds and determining its antioxidant and antidiabetic potential. The protein content was found to be 100.48 ± 0.72 mg/mL. The electrophoretic profile of pumpkin seed protein showed three distinct bands and the molecular weight ranged between 25 and 45 kDa. Results of antioxidant assays indicated that pumpkin seed protein possessed good antioxidant activity. IC₅₀ value refers to the amount required to scavenge 50% of free radicals. Smaller the IC₅₀ value, greater is the antioxidant power. Among the free radicals scavenged, pumpkin seed protein effectively scavenged ABTS^{•+} radical (80.87 ± 0.36% at 10 µg/mL) when compared to its ability to scavenge DPPH[•] radical (61.84 ± 1.10% at 1000 µg/mL) (Table 1). The IC₅₀ value of DPPH[•] radical and ABTS^{•+} radical scavenging assay was 670 µg/mL and 3.49 µg/mL. The antioxidant activity of food proteins and peptides depends upon the amino acid composition. Amino acids such as alanine, proline and phenylalanine have strong antioxidant properties.

Table 3: Alpha amylase inhibition assay of pumpkin seed protein.

| Concentration µg/mL | % inhibition of alpha amylase | |
|------------------------|-------------------------------|----------------------------|
| | Pumpkin seed protein | Acarbose |
| 20 | 4.04 ± 0.87 ^a | 49.68 ± 0.79 ^a |
| 40 | 18.35 ± 2.06 ^b | 51.72 ± 1.07 ^{ab} |
| 60 | 25.38 ± 2.61 ^c | 52.36 ± 0.40 ^{bc} |
| 80 | 41.55 ± 1.36 ^d | 54.23 ± 0.53 ^{cd} |
| 100 | 43.37 ± 0.82 ^d | 55.78 ± 0.41 ^d |
| 120 | 46.90 ± 0.53 ^d | 59.31 ± 1.05 ^e |

Values are the mean of triplicates

Different superscripts in a column are significantly different ($p < 0.05$).

Aromatic amino acids such as tryptophan and tyrosine function as free radical scavengers by donating a proton.^[23,24] Histidine functions as a strong antioxidant agent through different mechanisms such as scavenging hydroxyl radicals, quenching single oxygen, acting as metal chelating agents and by inhibiting the formation of peroxides.^[25] Kim *et al.*^[26] reported the protein content of pumpkin seeds (*Cucurbita maxima* L.) to be 274.85 ± 10.04 g/kg raw weight. Amino acids such as alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, lysine, isoleucine, methionine, tyrosine, proline and phenylalanine were present in pumpkin seeds. Upon quantification of amino acids, the authors of the study reported the content of alanine to be (10.16 ± 0.64) , histidine (16.51 ± 2.21) , phenylalanine (13.14 ± 1.23) , glutamic acid (48.94 ± 3.55) , valine (15.25 ± 0.78) and proline (10.05 ± 1.68) mg/kg raw weight. Presence of different amino acids in the protein sequence of pumpkin seeds significantly contribute to antioxidant activity. Fan *et al.*^[27] reported that protein isolates of pumpkin seeds (*Cucurbita pepo* L.) exhibited strong antioxidant activity. Similarly, Elham *et al.*^[28] also reported that protein extracted from *Cucurbita pepo* seeds also possessed strong antioxidant activity. Relationship between antioxidant activity and composition of amino acids was well documented in the study. Furthermore, bioactive proteins extracted from food exhibit antidiabetic potential by effectively inhibiting the action of digestive enzymes involved in starch breakdown. In the present study, pumpkin seed protein inhibited the action of alpha amylase ($46.90 \pm 0.53\%$ at $120 \mu\text{g/mL}$) and the IC_{50} value was $127.94 \mu\text{g/mL}$. Pumpkin seed can be used as a dietary supplement or as a functional food for treating hyperglycaemia. The hypoglycaemic effect of pumpkin seeds is evident through the results obtained from *in vitro* and animal model studies. Pumpkin seeds function as a hypoglycaemic agent by increasing hepatic

glycogen content, decreasing gluconeogenesis and by inhibiting the action of enzymes involved in starch breakdown.^[29,31] The results of the study point out that proteins present in pumpkin seed also contribute to antidiabetic activity.

CONCLUSION

The present study highlights that proteins present in pumpkin seeds exhibit antioxidant and antidiabetic potential. Hence plant proteins can be used as a functional food component. However, more research studies are needed on finding out the efficient method of protein isolation, amino acid sequence, bioavailability, *in vivo* health promoting effects of plant proteins and its usage in food industries as health promoting compounds.

ACKNOWLEDGEMENT

Authors thank the Director, Armats Biotek Training and Research Institute, Guindy, Chennai, Tamil Nadu, India for the facilities provided.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

DPPH•: 2, 2-diphenyl-1-picrylhydrazyl; **ABTS•+**: (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)); **SDS-PAGE**: Sodium dodecyl sulphate –polyacrylamide gel electrophoresis; **mM**: millimolar; **w/v**: weight by volume; **v/v**: volume by volume.

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Cite this article: Monica SJ, John S, Saraswathi K, Madhanagopal R, Arumugam P. Evaluation of Antioxidant and Antidiabetic Potential of Crude Protein Extract of Pumpkin Seeds (*Cucurbita maxima* L.). *Asian J Biol Life Sci*. 2020;9(2):152-7.