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Impact of Maturity Stage on Free Radical Scavenging and Antidiabetic Activities of Melinjo (*Gnetum gnemon* L.) Seed Proteins

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ABSTRACT

Seed storage proteins are a major protein source due to their readily available bioactive peptides. Melinjo (*Gnetum gnemon* L) seeds have a promising potential for massive production in Indonesia because of their high protein content. The composition and protein content of these seeds depends on their stage of maturity. This study investigated the effect of melinjo seed proteins at green (GG), yellow (YG), and red (RG) stages of maturity on their antioxidant and antidiabetic activities. Also, this study aimed to determine seed maturity's impact and identify the seeds most active stage on free radical scavenging (antioxidant) and antidiabetic activity. This study analyzed the amino acid composition, protein profiles, free radical scavenging, and in vitro antidiabetic activities of GG, YG, and RG seed proteins. The concentration of amino acids in melinjo seed samples was 0.36-9.69 g/100 g protein, with the total amino acid content in GG seeds (59.92 g/100 g protein) being significantly higher than in YG (53.91 g/100 g protein) or RG (52.79 g/100 g protein) seeds. The protein from GG seeds also exhibited significantly (p<0.05) higher free radical scavenging and in vitro antidiabetic activities than YG or RG seeds. The free radical scavenging activities were measured using ABTS, hydroxyl radical, and superoxide radical assays. The antidiabetic activity was assessed based on α-amylase and α-glucosidase inhibitory activities. The results indicated that the maturity stage of the seed proteins significantly affected (p<0.05) free radical scavenging and *in vitro* antidiabetic activities. GG seed protein showed high potential as an antioxidant and antidiabetic agent, suggesting its possible use in future nutraceuticals and human health applications.

Keywords: Maturity, Melinjo Seed, Antidiabetic, Antioxidant, Protein.

Introduction

Diabetes is a chronic metabolic disorder and a major global health issue due to elevated blood sugar levels.[1]This condition is often linked to the activities of α-amylase and α-glucosidase, key enzymes in the digestive process that convert starch into glucose.[2] The role of α-amylase in increasing glucose levels is particularly problematic for individuals with diabetes. Therefore, one therapeutic approach to managing diabetes involves inhibiting these enzymes to prevent the excessive accumulation of glucose in the blood. Specifically, inhibiting α-glucosidase can help reduce postprandial blood glucose levels[3] by delaying the breakdown of oligosaccharides and disaccharides into monosaccharides.[4] High blood sugar levels can also lead to an increase in free radicals, which cause cellular damage[5] and oxidative stress, further complicating diabetes and leading to various complications.[6] Antioxidants are crucial in preventing oxidative stress and mitigating its harmful effects.

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Recently, there has been significant interest in natural antioxidants derived from food and medicinal products.[7] Natural antioxidants can neutralize free radicals,[8] protecting the body from damage to cell components,[9] including DNA, lipids, and proteins, and enhancing the body's antioxidant defense system.[10] This interest extends to antioxidant proteins, which have shown promise as synthetic antioxidant materials. Proteins serve various biological functions, including acting as antioxidants by counteracting the harmful effects of free radicals[11]. Several plants, including the melinjo tree, have been identified as potential sources of antioxidant proteins. The melinjo tree (*Gnetum gnemon* L.), belonging to the Gnetaceae family (Gymnosperm), is cultivated in several regions of Indonesia, such as Sumatra and Java Islands.[12] Melinjo is commonly used as a vegetable and in the preparation of cakes and crackers. Melinjo seeds are notably rich in crude protein at16-19% (w/w).[13] Siswoyo *et al.*[14] demonstrated that melinjo seed protein extract possesses high antioxidant potential and can effectively scavenge various free radicals. Additionally, protein hydrolysis of melinjo seeds can enhance their antioxidant capacity and ability to protect against oxidative DNA damage.[14,15] Given these findings, further development of melinjo as a dietary and nutraceutical component is warranted. The use of natural substances in nutraceutical development has gained traction as an alternative to chemical drugs, which can have adverse side effects. Examples of such natural substances include hemp seeds,[16] *Erythrina edulis* (Pajuro) seeds[17] and sesame seeds,[18] all of which have high antioxidant potential. However, there is currently limited information regarding the antioxidant and antidiabetic activities of melinjo seed proteins at different stages of maturity. This study aims to investigate the impact of seed maturity on

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the antioxidant and antidiabetic activities of melinjo seed proteins and to identify the most active stage to maximize their potential.
The findings could pave the way for using these proteins as nutraceutical compounds to enhance human health.

Materials and Methods

Materials
The reagents used in this study were as follows: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), trinitrobenzene sulfonic acid (TNBS), bovine serum albumin (BSA), 2-deoxy-D-ribose, pyrogallol, 3,5-dinitro salicylic acid (DNS), ferric chloride solution ($FeCl_3$), hydrogen peroxide (H_2O_2), tertiary butyl alcohol (TBA), trichloroacetic acid (TCA) ethylenediaminetetraacetic acid (EDTA), α-amylase (30 U/mg) and α-glucosidase (10 U/mg). All reagents were procured from Sigma-Aldrich, Singapore.

Plant Collection and Identification
Melinjo seeds at various stages of maturity, including green (GG), yellow (YG), and red (RG), were obtained from an experimental farm at the University of Jember (8.1629° S, 113.7173° E), East Java, Indonesia, in April 2019 (Figure 1). Samples were identified and validated by the Laboratory of Plant Analysis and Botany, Faculty of Agriculture, University of Jember, with voucher number AGR01/24.

Protein Extraction and Amino Acids Analysis
Protein extraction from melinjo seed samples was performed by grinding 1 g of fresh seed with 3 mL of 0.1 M Na_2HPO_4 (pH 7.0) and centrifuging at 10,000 rpm for 15 minutes at 4 °C. The Bradford method[19] was used to determine soluble protein from the pooled supernatant. Absorbance was quantified by spectroscopy (Hitachi U-2900 UV/VIS, Tokyo, Japan) at 595 nm, and results were compared with a BSA standard. Total nitrogen content was calculated using the Kjeldahl method. Amino acid content was analyzed using method developed by Bhat *et al*.[20] Amino acid hydrolysis was conducted by weighing 0.1 g of each sample and adding 5 mL of 6 N HCl. Hydrolysis was carried out at 110 °C for 22 hours. The mixture was then cooled to 26-27 °C, transferred to a 50 mL measuring flask, diluted to the mark with deionized water, and filtered through a 0.45 µm filter. The α-aminobutyric acid (0.4 mL, 50 mM) was added as an internal standard. A 20 µL aliquot of hydrolysate was injected into a UPLC system (Waters 2475, US) using an AccQ. Tag Ultra C18 column µm (2.1×100 mm) with a Photodiode Array (PDA) detector set at 260 nm to identify amino acid compositions.

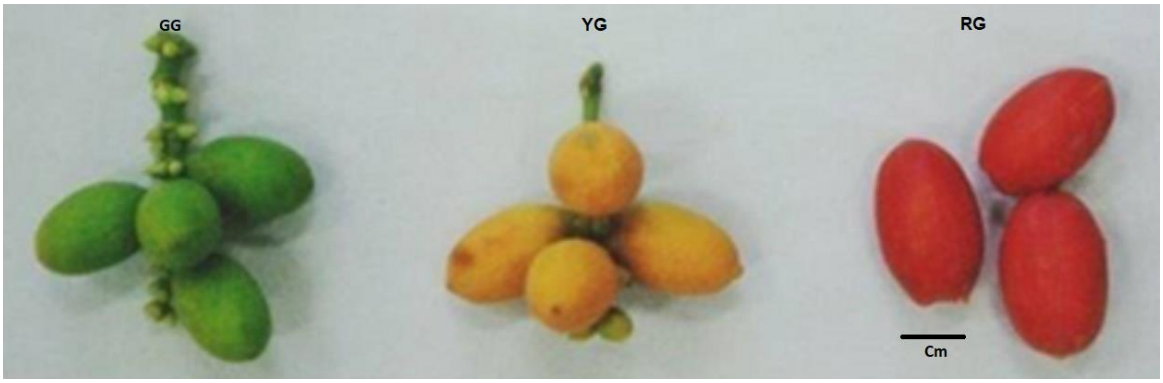

Figure 1: Melinjo seeds are in different stages of maturation: GG (green), YG (yellow), and RG (red).

Protein Profiling by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
The profiling of protein samples was done by loading 10 µg of protein sample into a 15% polyacrylamide gel.[21] Densitometry of various analyte proteins and their respective loading controls from the same band was performed using ImageJ software (Java 1.6.0_20). Relative optical density was calculated by dividing the densitometry of analyte(s) protein with the respective loading control, and the molecular weight was estimated using a protein marker (Thermo Scientific, Lithuania).

Antioxidant Activity Assay
The antioxidant activity was measured based on ABTS, hydroxyl, and superoxide radical scavenging activities. The ABTS radical scavenging assay was conducted using the method described by Re *et al*.[22] Antioxidant activity was calculated using the following formula:

$$ABTS\ (\%) = \langle [Ac - As] | Ac \rangle\ x\ 100\% \qquad /1/$$

Where Ac is the absorbance of the control and As is the absorbance of the sample. Hydroxyl radical scavenging activity was analyzed using the protocol described by Halliwell *et al*.[23] Superoxide anion radical scavenging was analyzed using the method of Tang *et al*.[24] Glutathione (G-SH) was used as a positive control for antioxidant activity.

Antidiabetic Activity Assay
The antidiabetic activity of melinjo seeds was analyzed based on the α-amylase and α-glucosidase inhibitory assays. The α-amylase inhibition assay, with slight modifications, followed the DNS procedure according to Awote *et al*.[25] 100 µL sample was added to 0.1 U/mL α-amylase in 150 µL of 20 mM Na_2HPO_4 (pH 6.9). The solution was preincubated for 15 minutes at 37 °C, then 250 µL of soluble starch (1%, w/v) was added. The mixture was incubated for another 15 minutes at 37 °C. The hydrolysis reaction was stopped by boiling the solution for 1 minute. To determine the reduction in total sugars, 50 µL of DNS reagent was added, and absorbance was measured with a microplate reader at 540 nm.
The α-glucosidase inhibition assay was performed using the method by Miyazawa *et al*.[26], with slight modifications. A 200 µL sample was added to 100 µL of 0.25 M maltose, 190 µL of 0.1 M KH_2PO_4 (pH 7.0), and 10 µL of α-glucosidase (1 U/µL) and incubated at 37 °C for 1 hour. The reaction was stopped by boiling the solution for 3 minutes. A 235 µL portion of the mixture was transferred to a new container, to which phenol buffer, (pH 7.0, containing 0.1 N KH_2PO_4, 3 mg/mL Triton-X and 3 mg/mL phenol solution), 5 µL of peroxidase (0.5 U/µL), 5 µL of glucose oxidase (0.8 U/µL) and 5 µL of aminoantipyrine (1 mg/mL) were added. The mixture was incubated at 37 °C for 10 minutes, and absorbance was recorded at 500 nm. Acarbose was used as a positive control.

Statistical Analysis
All experimental results were analyzed statistically using SPSS 17.0 software, with standard deviation (±SD) and analysis of variance (ANOVA). Significant differences were determined by Duncan's test at $p<0.05$.

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Results and Discussion

The protein content of the melinjo seeds in this study was 16–19% (w/w). Bhat and Yahya[13] reported a similar range, which is much higher than wheat and comparable to studies on wild and underutilized legumes, with values between 8.55% and 12–20%.[27] The high protein content in melinjo seeds underscores their potential as a significant nutrient source. However, it is important to note that protein content can vary depending on factors such as plant variety, agronomic practices, seed maturation stage, and the climatic and geological conditions of the area where the seeds are collected.[28] The stage of maturity affects the amino acid composition in the seed samples. Table 1 shows that in all samples, concentrations of Glu and Arg were the highest, whereas Met and Cys concentrations were relatively low. The variability in Pro concentrations among the GG, YG, and RG samples was relatively high, with a CV of 22.8%. Additionally, the concentrations of Lys, Arg, Asp, Phe, and Cys among the samples were moderately high, as indicated by the CV values. Table 2 shows the total nitrogen content, total amino acid (TAA), the total antioxidant amino acid grouping (TAntAA), and ratios at different stages of the melinjo seed maturation. The amino acid composition in melinjo seeds was presented as TAA. The different compositions of amino acids in the melinjo seeds (GG, YG, and RG) reflected varying amounts of TAA. GG seeds contained more TAA than YG or RG, ranging from 10–14%.

Table 1: The amino acid content (g/100 g protein) of melinjo (*Gnetum gnemon*) seed proteins at various stages of maturity

Components	GG	YG	RG	Mean	±SD	CV (%)
Aspartic (Asp)	5.30	4.24	4.87	4.80	0.53	11.11
Threonine (Thr)	3.01	2.73	2.49	2.74	0.26	9.33
Serine (Ser)	3.64	3.57	3.25	3.49	0.21	6.02
Glutamic (Glu)	9.69	8.08	8.68	8.82	0.81	9.20
Proline (Pro)	3.48	2.40	2.39	2.76	0.63	22.80
Glycine (Gly)	3.28	2.94	3.01	3.08	0.18	5.77
Alanine (Ala)	3.06	2.97	2.74	2.92	0.16	5.62
Cysteine (Cys)**	0.44	0.41	0.36	0.41	0.04	10.34
Valine (Val)	3.68	3.54	3.20	3.47	0.25	7.10
Methionine (Met)**	0.61	0.71	0.63	0.65	0.05	7.78
Isoleucine (Ile)	2.33	2.43	2.12	2.29	0.16	6.82
Leucine (Leu)	4.33	4.32	4.02	4.22	0.17	4.10
Tyrosine (Tyr)**	4.10	3.38	3.69	3.72	0.36	9.72
Phenylalanine (Phe)	2.29	2.86	2.57	2.58	0.28	11.00
Histidine (His)**	0.99	1.14	1.10	1.08	0.07	6.93
Lysine (Lys)**	3.64	2.87	2.83	3.11	0.46	14.80
Arginine (Arg)**	6.03	5.32	4.83	5.39	0.61	11.24
TAA (g/100 g protein)	59.92	53.91	52.79	55.54	3.38	6.90

*Values are reported as the mean of three separate determinations (n=3), **antioxidant amino acid grouping, SD=standard deviation, CV=coefficient of variation

Table 2: Average amino acid groupings of melinjo (*Gnetum gnemon*) seed proteins at various stages of maturity and their ratios

	GG	YG	RG	Mean	±SD	CV (%)
TAA (g/100 g protein)	59.92	53.91	52.79	55.54	3.38	6.90
TAntAA (g/100 g protein)	18.83	13.82	13.44	15.36	3.01	19.58
TAntAA/TAA (%)	31.43	25.64	25.46	27.51	3.39	12.34
N-Total (%)	2.54	2.42	2.19	2.38	0.18	7.57

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TAA levels were influenced by the total nitrogen content of the melinjo seeds. TAA in the samples was dominated by Cys, Lys, Arg, Met, Tyr, and His composition, similar to the antioxidant amino acid group reported by Xu *et al*.[29] The amount of those antioxidant amino acids is presented as TAntAA. Like the TAA value, GG seeds contained higher TAntAA than YG or RG, ranging from 27–29%. This is also reflected in the TAntAA/TAA ratio, with GG having a ratio of 18–19%, higher than YG or RG samples. The high TAntAA/TAA ratio of GG also contributed to the high variability value of TAntAA/TAA among GG, YG, and RG, with a CV of 12.34%. With its high TAntAA/TAA ratio, GG protein exhibited more potent antioxidant activity than YG or RG. SDS-PAGE profiling was conducted to characterize the protein with antioxidant potential based on its molecular weight. As shown in Figure 2, GG, YG, and RG have two major protein patterns with molecular weights of approximately 30 and 12 kDa. The GG protein at 12 kDa was 51% higher than YG and 14% higher than RG. Meanwhile, the GG protein at 30 kDa was 72% lower than YG and 65% lower than RG. Siswoyo *et al*.[14] reported that the amino acid composition of the 30 and 12 kDa proteins had the lowest Cys content but the highest Glu content. Regarding the antioxidant amino acid grouping, the 12 kDa protein fraction showed the highest content of Tyr and Lys. According to Pownall *et al*.,[30] Try and Lys play specific roles in enhancing the protein's antioxidant properties. Based on their amino acid composition, the 12 kDa protein fraction is estimated to have the highest antioxidant capacity compared to the 30 kDa protein fraction. Similar observations were reported for melinjo seed protein fractions, where the 12 kDa protein fraction exhibited higher antioxidant capacity than the 30 kDa protein fraction.[14] Peptide mass fingerprinting with MALDI-TOF MS revealed that the 30 and 12 kDa protein fractions differed significantly, and the MASCOT search program indicated that no protein in the database matches the 30 kDa or 12 kDa fractions, suggesting that these proteins are newly discovered and not widely characterized.

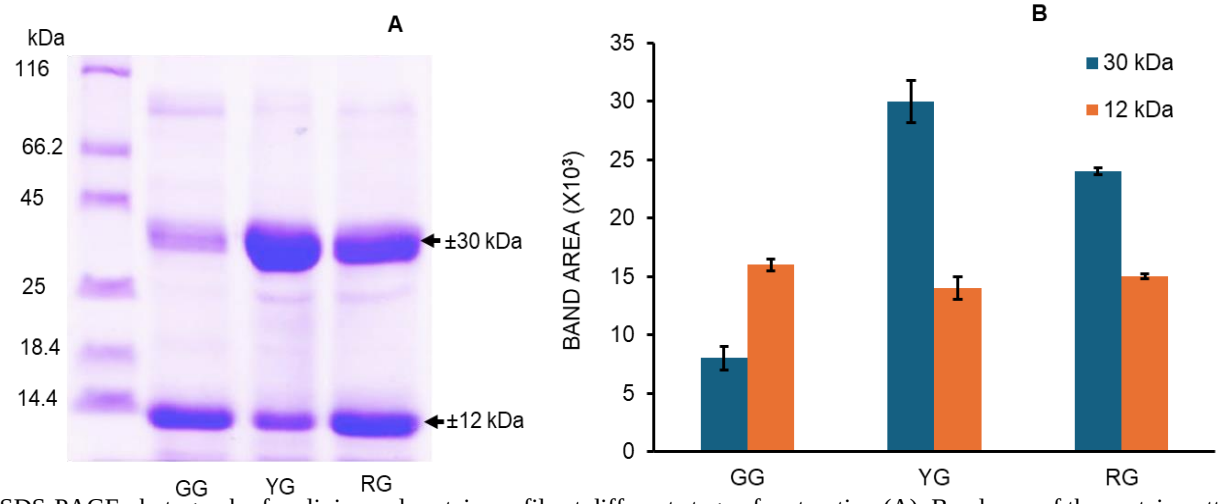
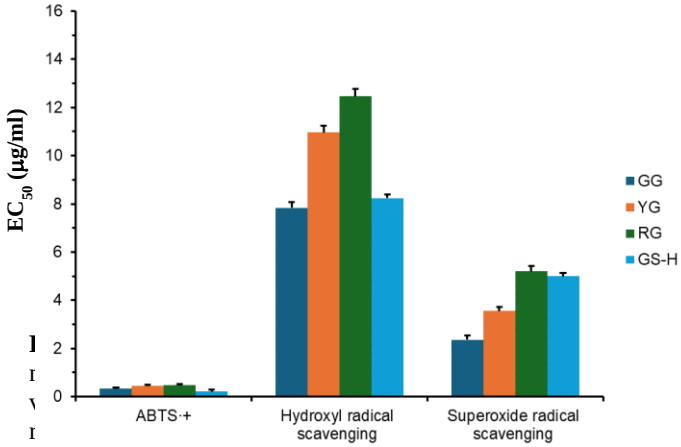


Figure 2: SDS-PAGE photograph of melinjo seed protein profile at different stage of maturation (A); Band area of the protein pattern analyzed using ImageJ software (B). GG (green), YG (yellow), RG (red).

Antioxidants play a pivotal role in preventing cellular damage caused by exposure to free radicals.[31] The GG protein extract demonstrated the highest antioxidant activities based on ABTS, hydroxyl radical, and superoxide radical scavenging, as shown in Figure 3. This high antioxidant activity is characterized by lower EC_{50} values in the GG protein extract. The ABTS radical scavenging activity of the GG protein extract has an EC_{50} value of 0.33 µg/mL, which is lower than the values for YG or RG (EC_{50} = 0.45 and 0.47 µg/mL, respectively). For hydroxyl radical scavenging, GG has an EC_{50} value of 7.83 µg/mL, lower than YG (10.96 µg/mL) and RG (12.46 µg/mL). GG has an EC_{50} value of 2.36 µg/mL for superoxide radicals, compared with YG (3.55 µg/mL) and RG (5.20 µg/mL). All antioxidant assay results were under 10 µg/mL (Figure 3). Shori[32] reported that natural resources with EC_{50} values lower than 500 µg/mL have a promising potential as nutraceutical resources and antidiabetic agents. The high antioxidant potential found in GG seeds correlates with their dominant antioxidant amino acid composition compared to YG or RG. Sochor *et al*.[33] stated that antioxidant amino acids could interact with free radical compounds, contributing hydrogen atoms to lipid radicals and allowing free radicals to stabilize.[34] This interaction shifts the equilibrium between antioxidants and free radicals.[35] The formation of free radicals contributes to diabetes complications, necessitating antioxidants to counter these effects through the metabolic inhibition of *α*-amylase and *α*-glucosidase. The IC_{50} values of *α*-amylase and *α*-glucosidase inhibitory activities of the GG protein were significantly higher (p<0.05) than those of YG or RG proteins (Figure 4). The IC_{50} values of *α*-amylase inhibition from the GG, YG, and RG proteins were 18.68, 35.46, and 36.10 µg/mL, respectively. The IC_{50} values of *α*-glucosidase inhibition activity from the GG, YG, and RG proteins were 10.86, 32.88, and 35.78 µg/mL, respectively. Both the *α*-amylase and



and superoxide radical. The values are presented as the means followed by standard deviations and analyzed using Duncan's test at p<0.05, Different letters within a group indicate significant differences.

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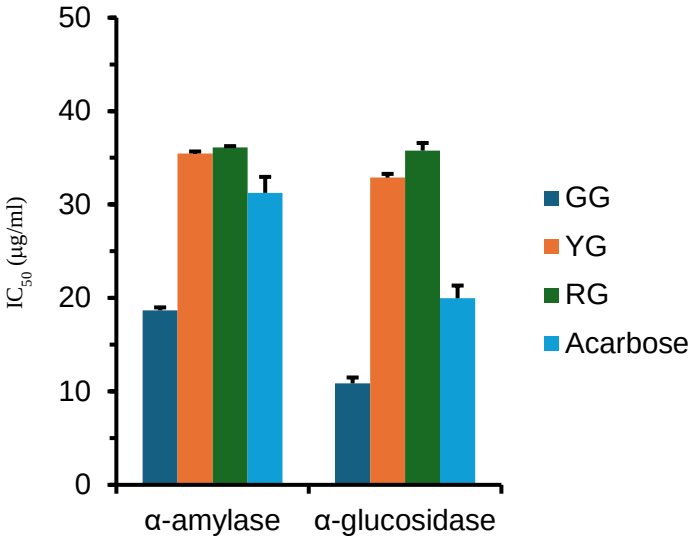

Figure 4: IC_{50} values for inhibiting α-amylase and α-glucosidase activities by melinjo seed proteins at various stages of maturity. IC_{50} value represents the concentration of protein required to inhibit 50% of the activity of α-amylase and α-glucosidase. The values are presented as means followed by standard deviations and analyzed using Duncan's test at $p<0.05$. Different letters within a group indicate significant differences.

α-glucosidase inhibition activities of GG protein were higher compared to acarbose, which had IC_{50} values of 31.25 and 19.97 µg/mL, respectively. These results suggest that GG protein has great potential for antidiabetic activities. The data indicate that melinjo seed protein can inhibit *α*-amylase and *α*-glucosidase activities. Recent findings have revealed that bioactive peptides from food sources can also inhibit the activities of both enzymes.[36] Protein peptides contribute electrons that bind *α*-amylase and *α*-glucosidase, thereby inhibiting their metabolic pathways.[37] These enzymes break down carbohydrates, necessitating carbohydrate inhibition to reduce postprandial glucose levels.[38] The findings provide experimental evidence that GG protein has a high potential for antioxidant and antidiabetic activities, suggesting its use in human nutraceutical health applications.

Conclusion

The present findings provide experimental evidence that ripening stages of seed proteins significantly affect ($p < 0.05$) free radical scavenging and antidiabetic activities. The green seed (GG) protein has high potential as an antioxidant and antidiabetic. This seed may serve as a candidate with the highest antioxidant and antidiabetic activities. The results obtained show that melinjo seed protein has a dual function as a free radical scavenging (antioxidant) and antihyperglycemic agent, making it promising nutritional material and bioactive source of antioxidants and antihyperglycemic peptide. Therefore, this protein might be used for human nutraceutical health applications.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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