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In vitro Antiglycation, Antioxidant and Antiproliferative Properties of Peptides Derived from Tryptic Hydrolysis of Soya Bean

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SSM, TAK, ALAM, MAZ and KOA designed the study. Author AK performed the study and the statistical analysis and wrote the manuscript. Authors OASB, MZ, NH and TMA reviewed the study. Authors MBM, FQS and FAM performed the antiproliferative assay. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Bioactive peptides are acknowledged for their vital contributions to health promotion. This study aims to evaluate antiglycation, antioxidant and antiproliferative ability of peptides derived from tryptic hydrolysis of Soya bean.

Place and Duration of Study: Department of Biochemistry, Department of Biological Science, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia, between September 2016 and June 2017.

Methodology: Hydrolysis profile was qualified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Reducing sugars-trapping ability of peptides was examined. 0.5% methacrylamido phenylboronic acid (MPBA) gel was used to investigate the advanced glycation end products (AGEs) inhibition. The in vitro antioxidant activity of peptides were tested by 2,2-diphenyl-1-picrylhydrazyl(DPPH) free radical scavenging assay. MCF-7, PC3, and HepG2 cell lines were used to explore the antiproliferative potency of peptides.

Results: Peptide fractions trapped reducing sugars and reduced AGEs formation, and exhibited well DPPH scavenging ability with IC_{50} 8.96 mg/mL. Peptide fractions showed dose-dependent toxicity to the used cell lines.

Conclusion: Released bioactive peptides emphasized the value of Soya bean proteins to be used as a functional food or alternative supplementary to diseases.

Keywords: Bioactive peptides; methacrylamido phenylboronic acid; antioxidant; antiglycation; antiproliferative; glycation.

1. INTRODUCTION

Numerous clinical investigations have highlighted the importance of raised advanced glycation end products (AGEs) in the human body as a risk factor for different diseases such as diabetes, and it`s complications and even for cancer [1,2,3]. On the other hand, elevated blood sugar level accelerates AGEs formation through glycation in which reducing sugars attack the free, reactive and functional amine group of proteins and impairs its functions [4]. These glycated proteins than, undergo rearrangement, degradation and crosslinking, finally become AGEs that contribute the reactive oxygen species (ROS) generation, another hallmark of diseases that interfere cellular signal transduction [5]. Therefore, AGEs inhibitors and breakers have been studied for decades. Because of the toxic effect of synthetic antiglycants, bioactive compounds, derived from different food sources, are becoming popular nowadays due to their health promotion, disease prevention ability [6]. Among them, bioactive peptides have been acknowledged for nutritional, functional and biological activities [7]. Bioactive peptides are protein fragments and inactive within the sequence of the parent protein and can be released by gastrointestinal digestion [8]. Since the publications reported biological activity of bioactive peptides including; anti-microbial; anti-viral; anti-fungal; anti-thrombotic; anti-hypertensive; immunomodulatory activities; cholesterol-lowering effect and antitumor activities; Protein-rich foods are consumed in large scale and regarded as a mainstream healthy choice [9].

Soya bean is one of the protein-rich foods and used as nutritional supplementation due to its reported biological activity. Wu et al. [10] studied the hypotensive activity of peptides derived from soy protein on spontaneously hypertensive rats. Also, many other peptide fractions were identified from soy protein hydrolysis with various specific biological activities such as hypocholesterolemic, anti-obesity, immunomodulatory and anti-microbial [11]. But limited studies were conducted about the antiglycation activity of soya proteins. In this study, we have investigated the in-vitro antiglycation activity, antioxidant and antiproliferative activity of soya bean protein hydrolysates.

2. MATERIALS

Soya bean was purchased from local market. HepG2, MCF-7, and PC3 cell lines were procured from King Fahd Medical Research Center, Jeddah, Saudi Arabia. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) were obtained from Hyclone Laboratories, Inc. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trypsin, bovine serum albumin (BSA), Trichloroacetic acid (TCA), Methacrylamido phenylboronic acid (MPBA) o-phthalaldehyde (OPA), 3,5-dinitrosalicylic acid (DNS), were obtained from Sigma-Aldrich Co(St. Louis, MO,

USA). Remaining chemicals used in this study were analytical grade.

3. METHODS

3.1 Preparation of Soya Bean Hydrolysate Samples

Soya bean protein was prepared by alkaline extraction followed by iso-electric precipitation at pH 4.2 as described by Lee et al. [12]. Briefly, Soya bean (100 g) were ground by a grinder then the flour was dewaxed by 500 mL hexane firstly and then mixed with 900 mL distilled water and pH was adjusted to 9.0 with 1M NaOH. The dispersions were stirred for 120 min at approximately 25℃ to accelerate protein solubilization, with the pH maintained at 9, and then centrifuged for 10 min at 4500 rpm at 25℃. The supernatant was collected and hydrochloric acid (0.1M) was used as an isoelectric precipitant (pH 4.2). The precipitate formed was centrifuged as described above. The pellet was dissolved in distilled water to remove nonprotein substances, the pH was adjusted to 4.2 and centrifuged. The pellet was then dissolved in distilled water with pH 7.9 and digested by trypsin E/S ratio 1:20 for overnight at 37℃, by shaking at 100 rpm. At the end of digestion, proteases were inactivated by heating the mixture for 15 min at 85℃. Soya bean hydrolysates were separated by centrifugation for 30 min at 4500 rpm, at 25℃, using ultrafiltration (UF) membranes (Amicon Ultra-4, Millipore, cutoff of 3 kDa), and permeate (less than 3 kDa peptides) was lyophilized. Digestion was qualified by SDS gel electrophoresis before and after digestion.

3.2 Peptides Ability to Trap Reducing Sugars

Peptides (0.5 mg/mL) were mixed with 30 mL of both 15 mM, 30 mM reducing sugars (glucose, fructose) in the phosphate buffer solution (pH 7.4). The sodium azide (0.002 wt %) was added to the mixture to inhibit microbial growth. The mixed solution was transferred to glass test tubes capped and stored in 37°C incubator for 22 days. During the storage period, sugar content and peptides concentration were measured. All model systems were prepared in triplicate.

3.2.1 Determination of sugars in reaction system

Reducing sugars concentrations in each system during the storage were measured using a 3,5-dinitrosalicylic acid (DNS) colorimetric method [13] with slight modification. Briefly, Sodium potassium tartrate solution was prepared by dissolving 135 g of sodium potassium tartrate in 225 mL of H_2O. DNS solution was prepared by dissolving 4.5 g of DNS reagent in 90 mL of 2 M NaOH. DNS reagent was prepared by mixing the sodium potassium tartrate solution and DNS solution to make up the volume to 450 mL with water and stored in the dark for one week before using. To determine the concentration of sugars, 0.5 mL sample solution was diluted with 0.5 mL water and mixed with 1 mL DNS reagent in the test tube. After heated for 5 min at 100°C, the mixtures were cooled down to room temperature. Finally, the mixture was diluted with 5 mL distilled water. The absorbance of the diluted solution and standards was measured at 540 nm against blank using a UV-Vis scanning spectrophotometer (Shimadzu UV-2101PC UV-VIS, Kyoto, Japan).

3.2.2 Measuring the peptides by o-phthalaldehyde (OPA)

The measurement of peptides concentration is based on their derivatization with OPA [14] to form a highly fluorescent adduct. Derivatizations of blank and sample solutions were performed by mixing 0.2 mL sample solution with 1.5 mL OPA solution complete and stirred thoroughly. After 2 min reaction at room temperature, the fluorescence intensity of OPA derivative solution was then recorded at an excitation wavelength of 348 nm and at emission wavelengths of 450 nm using Fluorescence Spectroscopy RF6000 (Shimadzu Corp, Japan). Spectral bandwidths of 2.0 and 10.0 nm were set for the excitation and the emission slits, respectively. The integration time in both was 1 s. The wavelength increment was 1 nm when running the spectrum scanning. The intensity of the spectra was determined as the emission signal intensity (counts per second) measured using a photomultiplier, and the average intensity of 15 sec was reported.

3.3 Antiglycation Activity

Antiglycation property of hydrolysates was evaluated by the method described by McPherson et al. [15] with slight modification. Bovine serum albumin (BSA) 5mL (10 mg/mL) prepared in phosphate buffered saline (PBS), pH 7.4, containing 0.002% (w/w) sodium azide was pre-incubated with peptides 0.5 mL (5 mg/mL)

dissolved in PBS (pH 7.4,) for 10 min at room temperature. 30 mM, 60 mM glucose, 30 mM fructose solution (5 mL) was added separately. The reaction mixture without glucose was used as a blank. The reaction mixture with glucose only was used as a control. The solutions were incubated in the dark at 37℃ for 22 days with gentle shaking.

3.3.1 Inhibition of AGEs formation

After incubation 1 mL 100% trichloroacetic acid (TCA) was added to reaction mixture, kept for 5 min to precipitate protein, centrifuged at 10000 rpm for 5 min. the pellet was washed with 5% TCA and dissolved in 2 mL PBS. AGEs formation was measured using a Fluorescence Spectroscopy RF6000 (Shimadzu Corp, Japan), with an excitation wavelength of 370 nm and an emission wavelength of 450 nm. The percentage of antiglycation activity was calculated using the following formula:

$$\text{Inhibition\%} = \{1-[(Ft - Fb)/(Fc - Fb)]\} \times 100$$

Where; Ft = fluorescence intensity of test sample, Fb = fluorescence intensity of blank, Fc = fluorescence intensity of control.

3.3.2 Qualifying BSA glycation using Phenyleboronate acrylamide gel electrophoresis

The incorporation of the specialized carbohydrate affinity ligand MPBA in polyacrylamide gels for SDS-PAGE analysis has been successful for the separation of post-translationally modified proteins [16]. 0.5% MPBA gel was prepared according to the method described by Morais et al. [17]. Briefly, 25 mg MPBA was added to the 5 ml, 12% resolving gel solution prior to polymerization and cast in cassette. After polymerization of the resolving gel, 4% stacking gel without MPBA was cast on the resolving gel. Glycated BSA (5 µL+5 µL loading buffer) was loaded to the stacking gel. The gel was electrophoresed at 60 mA for 90 min in glycine buffer at room temperature and stained with 0.25% Coomassie Blue R-250.

3.4 2,2-diphenyl-1-picrylhydrazyl(DPPH) Radical Scavenging Assay

The free radical scavenging property of hydrolysate was estimated according to the method described by Sharma, Om P. et al. [18]. Briefly, 200 mM DPPH reagent was prepared in 100 mL methanol, 1 mL of each hydrolysate was mixed with 1 mL of DPPH reagent. The reaction mixture was vortexed and incubated for 30 min in the dark at room temperature. The absorbance of the solution was measured at 517 nm. The ability to scavenge DPPH radical was calculated using the following equation:

$$\text{DDPH Scavenging effect (\%)} = [(Ac - At)/Ac] \times 100$$

Where; Ac = absorbance of control, At = absorbance of test.

Antiproliferative activity of soya bean peptides against cancer cell lines.

3.5 Cell Culturing

Cancer cell lines, MCF-7(breast cancer), PC3 (prostate cancer) and HepG2 (liver cancer) Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) with 10% fetal bovine serum (FBS, HyClone) and 1% antibiotics penicillin-streptomycin (HyClone). All the cell lines were maintained in CO_2 incubator with 37°C, 95% air humidified atmosphere and 5% CO_2.

3.5.1 Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

To assess the antiproliferative activity of Soya bean hydrolysate fractions on cancer cell lines, MTT assay was performed. Briefly, 8000 cells were seeded in each well of 96-well flat bottom plate and treated with the peptide after 24 hours. MTT assay was performed after 48 hours of treatment with the peptide. To perform the assay, 10 µl of MTT solution was added to each well and incubated plate for 3 hours. Later media containing MTT was aspirated, and 100 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple formazan. After 10 minutes of adding DMSO, the purple color intensity was measured at 540 nm by using the micro-plate reader (BioTek Synergy).

3.6 Statistical Analysis

All samples were prepared and analyzed in triplicate. All the data obtained were expressed as mean ± SD. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) test using GraphPad Prism 7. $P<0.05$ was considered to be statistically significant.

4. RESULTS

4.1 Gel Electrophoresis before and after Digestion

SDS page result in Fig. 1 demonstrated that Tryptic digestion was successful.

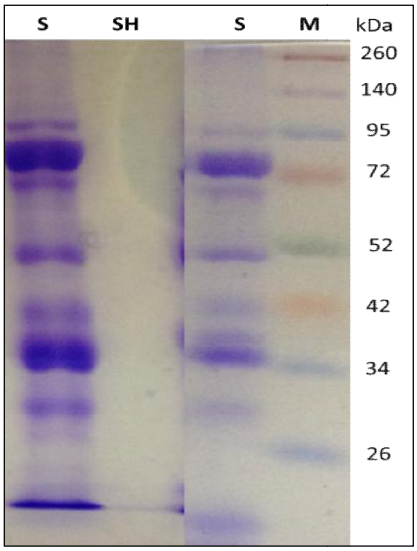

Fig. 1. Gel electrophoresis for 5 µl soya protein (17.37 mg/ml, approximately 86 µg) before and after digestion

S = Soya bean protein before digestion.
SH = Soya bean protein after digestion

4.2 Determination of Sugars in Reaction System

Reducing sugars bind with free amine groups in proteins and block its function. Peptides were incubated with 15 mM, 30 mM reducing sugars (glucose, fructose) to explore the peptides reactivity with reducing sugars. As shown in Fig. 2, Fig. 3 and Fig. 4, the sugars concentration was decreased day by day, on the day of 19 the reducing sugars concentration in all three reaction system with peptides was significantly reduced ($p<0.05$) compared to the control group. Fructose was more active than glucose in reaction with peptides, and the reaction was finished at 12th day.

4.3 Determination of Peptides by OPA

OPA test is used to determine the peptide concentration. OPA reacts specifically with primary amines to form fluorescent moieties. Results in Fig. 5 indicated that peptides were involved in the reaction and concentrations were decreased day by day. Peptide concentrations in three tested groups were significantly reduced on day 20 compared to the control group. In fructose reaction system, the reaction was stopped on day 10. It indicated that the reaction was fast and all the active peptides were consumed within ten days.

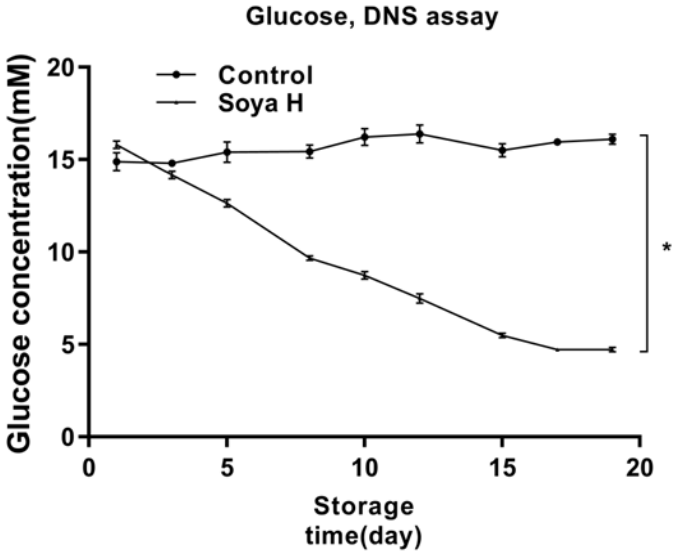

Fig. 2. Determination of glucose (15 mM) by DNS

Results are expressed as Mean ± SD, n=3 (Mean values ± Standard deviation of means of three experiments).
** represents $p<0.05$ when glucose concentration in Soya H group compared to the control group on day 19*

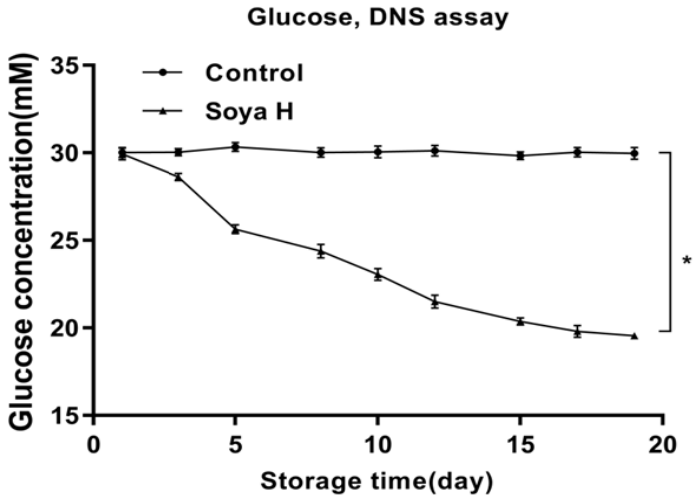

Fig. 3. Determination of glucose (30 mM) by DNS

Results are expressed as Mean ± SD, n=3 (Mean values ± Standard deviation of means of three experiments).
** represents p<0.05 when glucose concentration in Soya H group compared to the control group on day 19*

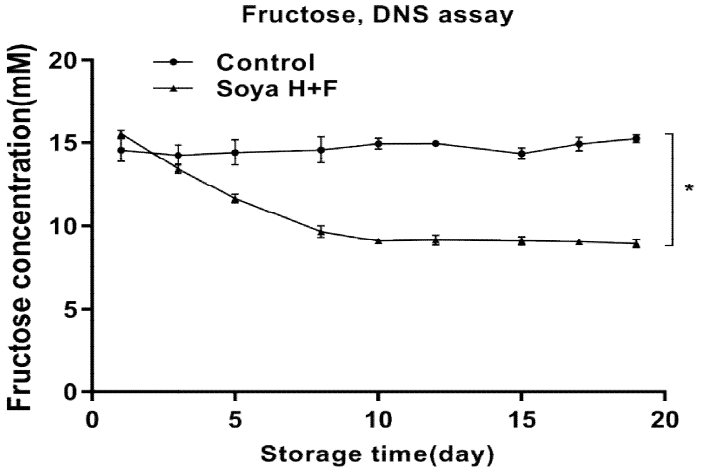

Fig. 4. Determination of fructose (15 mM) by DNS

Results are expressed as Mean ± SD, n=3 (Mean values ± Standard deviation of means of three experiments).
** represents p<0.05 when fructose concentration in Soya H group compared to the control group on day 19*

4.4 Inhibition of AGEs Formation

The formed of AGEs was determined by measuring the fluorescence intensity at the end of incubation. As shown in Fig. 6, compared to the control group BSA glycation was significantly increased in all other groups. The soya bean hydrolysate protected BSA from glycation, AGEs formation was significantly decreased in group D compared to the group B while the reduction was more in group F compared to group D. this indicates fructose is more active than glucose in glycation. Glycation was significantly reduced in group E compared to the group C. The reduction percentage in group E is similar with group D. it means reduction was dose dependent.

4.5 Qualifying BSA Glycation Using Phenyleboronate Acrylamide Gel Electrophoresis

MPBA-SDS gel method was developed for specifically separate glycated proteins from non-glycated proteins due to its affinity to carbohydrate modified proteins. It can be seen from Fig. 7; the black arrow indicates the nonglycated BSA while the white arrow indicates sugar modified BSA. More modified BSA are

visible in lane 2 and 3 compared to lane 4, 5 and 6. The less modified proteins in lane 6 match with the results of Fluorescent and Congo red binding assay.

4.6 Congo Red Binding Assay

The alteration of the secondary structure of BSA resulted in glycation was investigated by Congo red binding assay. Results in Fig. 8 demonstrated that compared to the group A, the secondary structure of BSA was significantly changed in group B, C ($p<0.001$) and group D, E, F ($p<0.01$). However, Soya bean hydrolysate significantly inhibited ($p<0.05$, group D and F compared to group B. group E compared to group C) the secondary structure alterations of BSA, compared to glycated BSA in different sugar level.

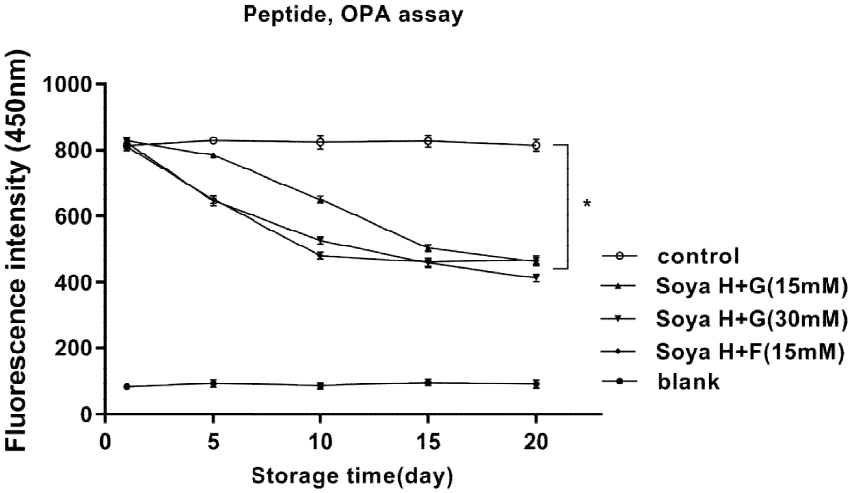

Fig. 5. Determination of the peptides in the reaction system by OPA

*Results are expressed as Mean ± SD, n=2 (Mean values ± Standard deviation of means of two experiments). * represents p<0.05 when the peptides concentrations in glucose and fructose added group compared to the control group*

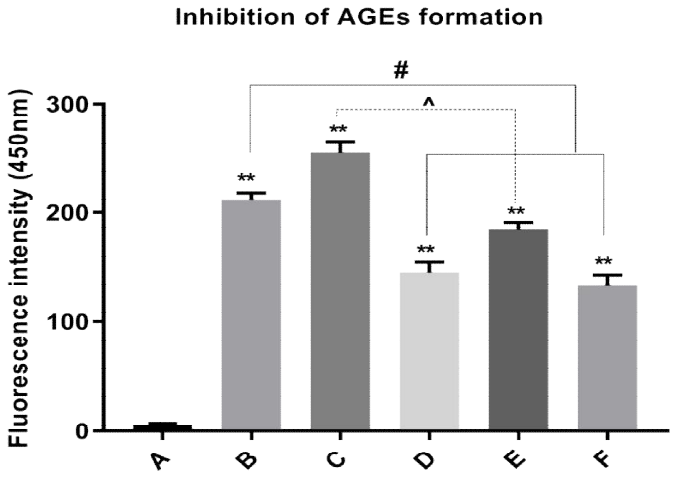

Fig. 6. Inhibition of AGEs formation by soya protein hydrolysate
A=BSA, B=BSA+15 mM Glucose, C=BSA+30 mM Glucose, D=BSA+15 mM Glucose + Peptide, E=BSA+30 mM Glucose +Peptide, F=BSA+15 mM fructose + Peptide

*Results are expressed as Mean ± SD, n=3 (Mean values ± Standard deviation of means of three experiments). ** represents p<0.01 while group B, C, D, E, F compared to the control group. # represents p<0.05 while group D, F, compared to the group B. ^ represents p<0.05 while group E compared to the group C*

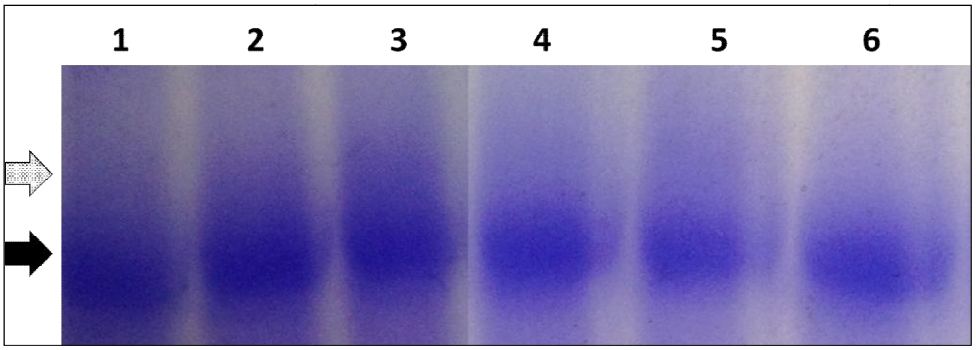

Fig. 7. 0.5% MPBA gel electrophoresis image
1=BSA, 2=BSA+15 mM Glucose, 3=BSA+30 mM Glucose, 4=BSA+15 mM Glucose + Peptide, 5=BSA+30 mM Glucose +Peptide, 6=BSA+15 mM fructose + Peptide, black arrow indicates glycated BSA band, white arrow indicates BSA band

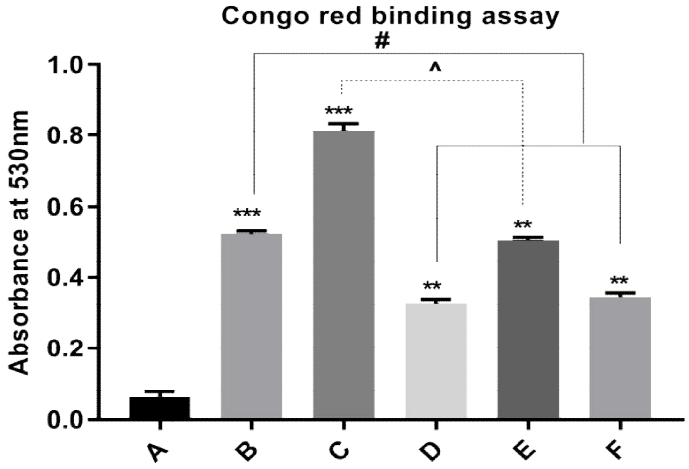

Fig. 8. Congo red binding assay
A=BSA, B=BSA+15 mM Glucose, C=BSA+30 mM Glucose, D=BSA+15 mM Glucose + Peptide, E=BSA+30 mM Glucose +Peptide, F=BSA+15 mM fructose + Peptide

*Results are expressed as Mean ± SD, n=3 (Mean values ± Standard deviation of means of three experiments). ** represents p<0.01 while group D, E, and F compared to the control group A. *** represents p<0.001 while group B and D compared to the group A. # represents p<0.05 while group D and F compared to the group B. ^ represents p<0.05 while group E compared to the group C*

4.7 DPPH Radical Scavenging Assay

The DPPH radical scavenging assay was applied to test the ability of Soya bean hydrolysate to neutralize DPPH. It can be seen in Fig. 9 that, different concentration of Soya bean hydrolysate was tested. Soya bean hydrolysate showed antioxidant activity to DPPH radical with an IC_{50} value of 8.96 mg/mL.

4.8 MTT Assay

Peptides from soya bean hydrolysate exhibited growth inhibitory effect to MCF-7, PC3, and HepG2 Cells. Cells were treated with the increasing concentration of peptides to study the anti-proliferative activity. As shown in Figs. 10, 11, 12. For MCF-7 and HepG2 the concentrations used for treatment were 5, 10, 15, 20 and 30 mg/mL and for PC3 0.5, 2.5, 5, 7.5 and 10 mg/mL was used. After 48 hours of treatment, the cell viability was measured using MTT. Dose dependent inhibition was clearly evident from the results of MTT assay in all three cell lines. The IC_{50} was calculated. The IC_{50} of MCF-7 is 9.84 mg/ml, PC3 is 3.05 mg/mL and HepG2 is 30.33 mg/mL

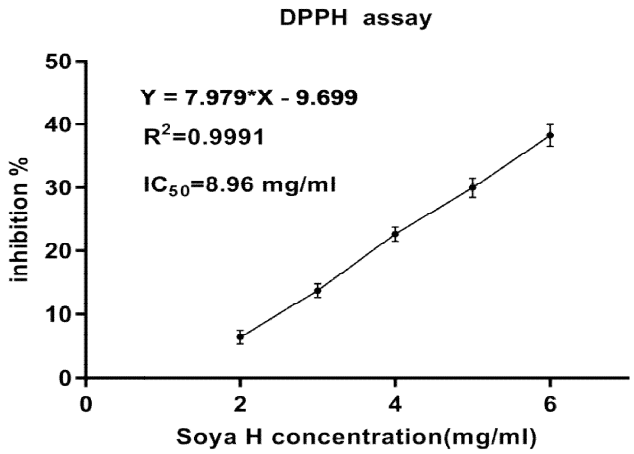

Fig. 9. DPPH radical scavenging assay. IC_{50}=8.96 mg/mL
Results are expressed as Mean ± SD, n=3 (Mean values ± Standard deviation of means of three experiments)

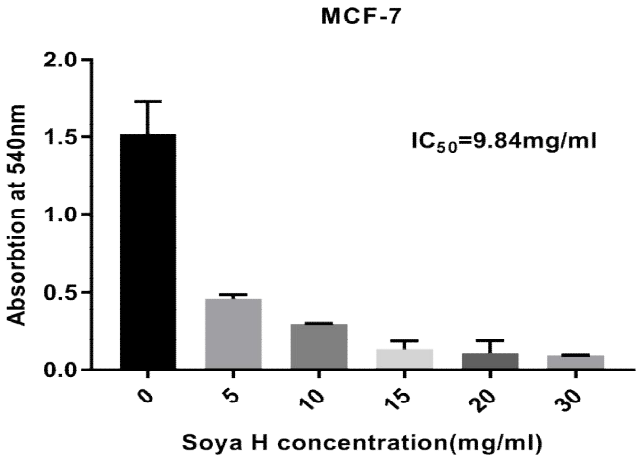

Fig. 10. MTT cell viability assay for MCF-7 cell line. IC_{50}=9.84 mg/mL
Results are expressed as Mean ± SD, n=2 (Mean values ± Standard deviation of means of two experiments)

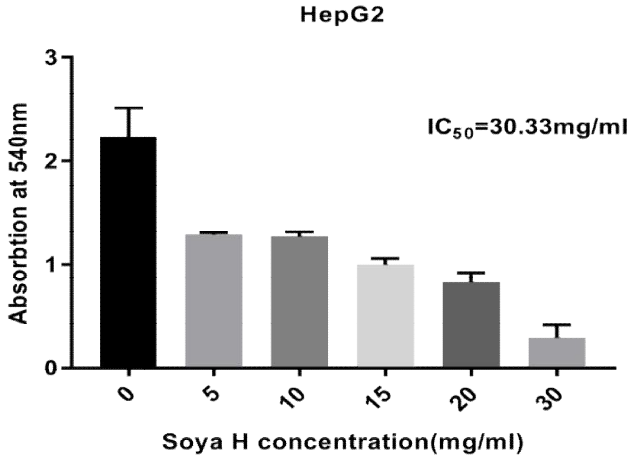

Fig. 11. MTT cell viability assay for HepG2 cell line. IC_{50}=30.33 mg/mL
Results are expressed as Mean ± SD, n=2 (Mean values ± Standard deviation of means of two experiments)

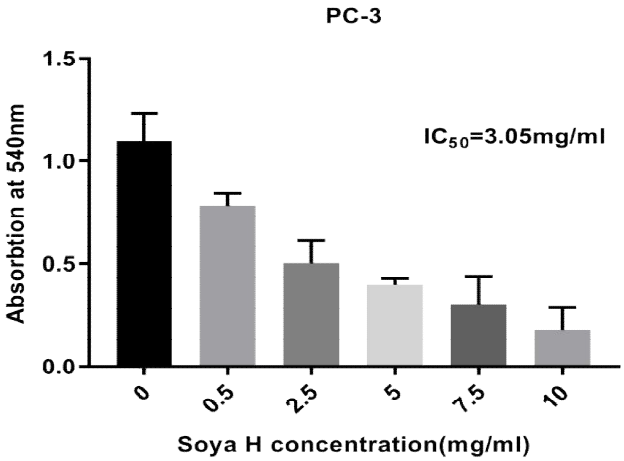

Fig. 12. MTT cell viability assay for PC3 cell line. IC_{50}=3.05 mg/mL

Results are expressed as Mean ± SD, n=2 (Mean values ± Standard deviation of means of two experiments)

5. DISCUSSION

AGEs have been recognized as hallmarks of various diseases such as diabetes, and its complications even for cancer [1,19,20]. It is generated by endogenous hyperglycemic and oxidative stress-related processes. The free and functional amine group of enzymes and structural proteins are susceptible to glycation and chronic hyperglycemia speed up the glycation processes [4], such as hemoglobin. The glycated hemoglobin leads an increase of ROS inside blood cells therefore alters blood cell membrane properties finally results in cell aggregation and increased blood viscosity which impair the blood flow [21]. Therefore, providing peptides with free and active amine group into the hyperglycemic condition is another therapeutic method for protecting functional macromolecules (DNA, Lipids and proteins) from glycation. It was reported that, the dipeptide Carnosine (0.01%) mixed with diet significantly suppressed hyperglycemia in diabetic rats [22]. In this study, we have prepared peptide fractions from soya bean by tryptic hydrolysis and evaluated the peptide fraction`s reactivity with glucose and fructose as well as antioxidant and antiproliferative potency. Results illustrated that soya bean peptides were active and significantly reduced sugars concentrations. This might be the exposure of active amine groups within the soya proteins by trypsin that cuts protein from lysine and arginine bond, and these amino acids are reported to involve in glycation. BSA antiglycation assay results indicated that soya bean peptides protected BSA from glycation by trapping extra sugars. BSA was prevented from further aggregation according to the Congo red binding assay results. Also, concentration-dependent antioxidant activity was observed in DPPH radical scavenging assay. Black Soya Bean was reported to host antioxidant peptides [23]. Wang et al. [24] separated novel antioxidant and ACE inhibitory peptide from rice bran protein. Chickpea, potato, and lentil were digested by different enzymes, and potential antioxidant and antiglycation peptides were identified [25,26,27].

In the antiproliferative assay, Soya bean protein hydrolysate showed cytotoxicity to MCF7, PC3, and HepG2 cancer cell lines in a dose dependent manner. This might be the antioxidant activity, or these peptides interfered the signal transduction of cancer cell lines. Researchers have explored an antiproliferative activity of purified single peptide from rice bran against breast cancer. They discovered that Caspase-dependent pathways were targeted by peptides to inhibit human breast cancer cell proliferation [28,29]. Some peptides with antioxidant activity exhibited antiproliferative activity [30]. Our results have indicated the potential of soya bean peptides in reducing the risks of different cancers. Further study is needed to explore the mechanism of antiproliferative activity of soya bean protein.

6. CONCLUSION

Keeping blood sugar level in normal clinical range is important to reduce the risk of diabetes and its complications. For this, natural bioactive compounds with antiglycation and antioxidant potential would be recommended. Numerous bioactive peptides from different sources have been identified. In this study tryptic hydrolysate of

soya bean was reported to have antiglycation, antioxidant and antiproliferative ability. It would be recommended as a functional food due to its high content of proteins and capability of releasing bioactive peptides by digestive enzymes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENT

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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