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Original Article

Inhibitory Effect of Bioassay-Guided Fractionation of Mushroom (*Pleurotus ostreatus)* Extract on Fructose-induced Glycated Hemoglobin and Aggregation *in vitro*

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ABSTRACT

Background: Chronic hyperglycemia in diabetes mellitus is a prominent inducer for free radical production which results in non-enzymatic protein glycation that cause the pathogenesis of diabetic complications and deleterious health-related issues. Since ancient times, people have consumed mushrooms as food and as a form of folk medicine. Mushrooms are becoming more and more popular due to their medicinal and nutritional advantages. *Pleurotus ostreatus* has considerable antioxidant properties to scavenge free radicals and is helpful to offer antiglycation activity. **Objectives:** The purpose of this research was to determine the inhibitory effect of bioassay-guided fractionation of *Pleurotus ostreatus* on formation of glycated hemoglobin and aggregation of glycated protein *in vitro*. **Materials and Methods:** Total phenolic and flavonoids were determined from the crude extract of *P. ostreatus*. Antioxidant capability of methanolic crude extract was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, H_2O_2 (hydrogen peroxide) scavenging, and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6 -sulfonic acid) radical cation scavenging activity. Inhibitory effect of bio-guided fractionations of crude *P. ostreatus* extract were measured using fructosamine formation, protein carbonyls, aggregation index, and fluorescence intensity. **Results:** Methanolic extract of *P. ostreatus* has significantly high levels of phenols (16.89 ± 1.44 mg GAE/g) and flavonoids (9.40 ± 0.86 mg QE/g). Methanolic extract was found to have excellent antioxidant capacity indicates from IC_{50} values for DPPH radical scavenging (75.23 ± 2.32 µg/mL), H_2O_2 scavenging (96.54 ± 2.32 µg/mL), and ABTS radical cation (50.33± 1.82 µg/mL) scavenging activities. The analysis on fructose induced glycated hemoglobin model revealed that fraction F4 of methanolic extract of *P. ostreatus* is more potent to inhibit formation of glycated hemoglobin by (58.84 ± 2.36%), fructosamine (64.32 ± 3.74%) and protein carbonyls (51.35 ± 2.94%). It also showed an aggregation index of 46.32 ±1.05% and a decline in fluorescence intensity (52.04%) which are important factor in many pathological processes. **Conclusion:** The fraction F4 obtained from *P. ostreatus* can be used for the identification of bioactive compounds that can be further considered as therapeutic agents for AGE-associated pathogenesis.

Keywords: Advanced glycation end products (AGEs), Anti-glycation, Antioxidant, HbA_{1c}, *Pleurotus ostreatus*.

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INTRODUCTION

A non-enzymatic interaction occurs between free amino groups of proteins molecules and the aldehyde group of reducing sugars (mostly hexose sugar like glucose, fructose, galactose, and also pentose sugar like ribose, etc.) and free amino groups of proteins molecules, referred to as glycation. In 1912, researcher Louis Camille Maillard founded this reaction, also known as the Millard reaction.[1] An elevated blood sugar level (hyperglycemia) persistent over long periods of time could enhance the rate of glycation of various physiologically important protein. The formation of advanced glycation end products (AGEs) takes place in three successive stages. A reversible reaction between sugar and protein makes Schiff bases, which are unstable. After a few weeks, the Schiff base undergoes rearrangements to form a relatively stable early glycation product called Amadori products. These products may be transformed to carbonyl components by dehydration, oxidation, or cyclization, and at the end, AGEs are formed. Glycated hemoglobin is an early-stage glycation adduct formed when glucose react non-enzymatically with amino

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group of N-terminal valine present on β-chain of hemoglobin used as a biomarker of glycemic control.[2] Glycation causes the modification of protein functions by the production of free radicals, cross-linking, aggregation, and precipitation.[3] In addition, AGEs not only interact with the AGE Receptors (RAGE) but with other ligands such as amyloid peptide (A), which forms agglomerates that lead to increased oxidative stress, inflammation, neuronal dysfunction, and Alzheimer's disease. Further, Protein glycation is associated with increased oxidative stress leading the pathogenesis of diabetes and its associated secondary complications, including cardiovascular and neurodegenerative disorders, cancer, and physiological ageing.[4] Occasionally, protein glycation causes enzymes to become inactive and cellular metabolism to become dysfunctional. Oxidative stress or carbonyl stress may specifically be attributed to the inactivation of antioxidant enzymes. Protein carbonyl stress is one of indicator of protein oxidation and it leads to cell damage that is associated with several human diseases.[5] To offer an effective therapeutic approach for diabetes and its comorbid problems, identifying suitable anti-hyperglycemic or anti-glycating medicines with natural origin has been the focus of extensive research.

Since ancient times, mushrooms have been utilized as a traditional medicine and as a food source. The nutritional value of oyster mushrooms comes from the proteins, carbohydrates, vitamins, minerals, high content of fibre, and low content of fat present in the fruit bodies.[6] *Pleurotus ostreatus* is the most widespread edible species among the *Pleurotus* species having nutrient-rich dietary composition.[7] *P. ostreatus* has various therapeutic properties such as antioxidant, anti-cancer, immune-modulating, anti-tumor, anti-arthritic, anti-inflammatory, hypocholesterolemic, anti-hyperglycaemic, anti-genotoxic, anti-hypertensive, anti-platelet aggregating, anti-viral, and anti-microbial activities.[8] *P. ostreatus* has considerable antioxidant and antidiabetic properties, as evaluated through *in vitro* and *in vivo* studies. In 2010, it was shown that a *P. ostreatus* extract improved the expression of the catalase gene and reduced the frequency of free radical-induced protein oxidation in elderly rats, reducing the development of age-associated diseases involving free radicals.[9] In addition to these functions, little is known about *P. ostreatus*'s antiglycation ability. Therefore, the current study was carried out to examine the antioxidant capacity of crude extract and the anti-glycation potential of bio-guided fractions of *P. ostreatus* using fructose-induced glycated hemoglobin.

MATERIALS AND METHODS

Collection and identification of mushroom species

P. ostreatus was bought for this study from B. A. College of Agriculture, Anand Agricultural University, Anand. The oyster mushrooms were identified on the bases of morphological characters (Average 12.5 cm wide mycelium; overlapping clusters; fan-shaped white pileus of mushrooms; gills are white; Whitish stipes of about 4.0 cm long in average).

Preparation of mushroom extracts

The harvested oyster mushrooms were ground into powder and dried for 24 hr at 45°C in hot air oven before being packaged in airtight containers. The dried mushrooms powder was kept at ambient condition for further investigation. The powder (20 g) was applied for extraction using solvents (hexane, butanol, distilled water, and methanol) through the Soxhlet apparatus individually. The extracts were collected and applied to a vacuum rotary evaporator. The residues that were left over after evaporation were weighed and dissolved in 10 mL of dimethyl sulfoxide, and they were used as crude extracts for further investigation. The purpose of using dimethyl sulfoxide is, it can dissolve both polar and nonpolar molecules present in residues. The current experiment used analytical-grade chemicals.

Estimation of Total Phenolic Content (TPC)

The amount of total phenolic content in crude extracts was analysed by a previously described method with some modification.[10] In brief, 0.1 mL of the extract was mixed with 0.2 mL of FCR (Folin-Ciocalteu reagent), 3 mL of distilled water, and 2 mL of Na_2CO_3 (20% w/v). A reaction mixture was kept in incubation at 100°C for 1 min. The absorbance was measured at 765 nm in the UV-visible spectrophotometer. The TPC was then calculated as the gallic acid equivalent per gram of dry powder.

Estimation of Total Flavonoid Content (TFC)

A method described by Marinova *et al.*[11] was used to estimate TFC. In brief, 0.1 mL of the extract was added to 0.1 mL of aluminium chloride (10% w/v) and 0.1 mL of potassium acetate (1M). The mixture was mixed thoroughly and kept at 25°C for 30 min, and the absorbance was taken at 415 nm using a UV-visible spectrophotometer. The TFC was then calculated as the quercetin equivalent per gram of dry powder.

Determination of total antioxidation activity

Free radical scavenging activity by DPPH

According to a previous approach, the extract's capacity to scavenge free radicals for DPPH (2,2-diphenyl-1-picrylhydrazyl) was evaluated with little changes.[12] For this estimation, 0.1 mL of extract at various concentrations was mixed with 1.0 mL of 0.1 mM DPPH reagent, and the mixture was thoroughly mixed. In addition, the reaction mixture was kept in the dark at room temperature for 30 min. The optical density was measured at 517 nm. In this assay, vitamin C was taken as a standard, and as a control, DPPH was used. The results were expressed in terms of inhibition using the formula given below:

$$\%\text{Inhibition} = [(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}] \times 100$$

Hydrogen peroxide scavenging activity

The capability of the extract to scavenge hydrogen peroxide was determined according to replacement titration method described by Zhang,[13] with some alterations. Two drops of 3% ammonium molybdate, 10 mL of 2.0 M H_2SO_4, and 7.0 mL of 1.8 M KI were then added to the mixture of 1.0 mL of 0.1 mM H_2O_2, 1.0 mL of extract with various concentration. 5.09 mM of sodium thiosulfate (NaS_2O_3) was titrated into the combined solution until the yellow colour disappeared. The formula used to determine the percentage of hydrogen peroxide scavenging is:

$$\% \text{ Inhibition} = [(V_0 - V_1)/V_0 \times 100]$$

Where V_0 denote the volume of sodium thiosulfate solution used to titrate the control sample in the presence of H_2O_2 (without extract), V_1 indicates the volume of NaS_2O_3 solution used in the presence of the extracts.

ABTS's radical scavenging activity

The extracts' ABTS radical-scavenging capacity was assessed using a technique described by Re *et al.*[14] with minute changes. It works on the ability of extract to reduce the radical cation $ABTS^+$ to ABTS'(2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). A free radical cation was produced by reacting 7.0 mM solution of ABTS in water with 2.45 mM potassium per sulphate ($K_2O_8S_2$) in a ratio 1:1. The combination was incubated at 27°C in the dark to achieve steady absorbance at 734 nm. After 16 hr of incubation, this radical ABTS reagent was further diluted with distilled water (1:25) to obtain the initial absorbance value of 0.7 at 734 nm. Now, 200 µL of the sample or standard (Ascorbic acid) was mixed with this 1.8 mL of $ABTS^+$ solution and allowed to react for 7 min. Absorbance was taken at 734 nm, where the $ABTS^+$ solution was used as a control. $ABTS^+$ scavenging inhibition capability was calculated as per the given formula:

$$\%\text{Inhibition} = [(\text{Absorbance of control-Absorbance of sample})/\text{Absorbance of control}] \times 100$$

Bio-guided fractionation of crude *P. ostreatus* extract

The column chromatography technique was used for the bio-guided fractionation of crude *P. ostreatus* extract. The column was packed with 20 gram of silica gel (60–120 mesh). To this bed of silica, the crude extract was added. Addition of the solvent to the column in an increased polarity order to accomplish fractionation with 100% hexane, hexane-ethyl acetate (50% each), ethyl acetate (100%), ethyl acetate-methanol (50% each), and 100% methanol was done. Five different fractions were collected (i.e., F1, F2, F3, F4, and F5) and assessed for anti-glycation activity.

Incubation of Bio-guided fractionations of crude *P. ostreatus* extract with *in vitro* glycation system

Glycated hemoglobin was prepared according to previously described procedure with minor changes to determine the inhibitory effect on protein glycation at an early stage.[15] Human hemoglobin was procured from Sigma Company. Hemoglobin (10 mg/mL) were mixed with fructose (500 mM) and incubated at temperature-controlled chamber (37°C) for 28 days in phosphate buffer (100 mM, pH 7.4) consisting of 3 mM sodium azide with and without all five fractionations of crude *P. ostreatus* extract in a concentration-dependent manner. Similarly, BSA (10 mg/mL) were incubated with sugar fructose (500 mM) in same experimental condition. BSA and Hemoglobin only considered as control blank and fractions of extract with respective concentrations are taken as sample blanks. Aminoguanidine (AG) was considered a positive control. The assessment of antiglycation activity was determined through the measurement of glycated hemoglobin, fructosamine level, protein carbonyl content, aggregation index, and fluorescence intensity at the end of experiment.

Methods for measurement of antiglycation potential

Estimation of glycated hemoglobin

The amount of glycated hemoglobin formed was estimated by semi-automatic HbA_{1c} Analyzer works based on Boronate Affinity Chromatography. Glycated hemoglobin containing cis-diol groups can bind to the Boronate column when sample passed through the column. The glycated hemoglobin is than eluted from the column that detaches from the Boronate and measure with spectrophotometric detector at 413 nm. HbA_{1C} level was calculated using the following formula:

$$\% \text{ } HbA_{1c} = HbA_{1c}/\text{total hemoglobin X } 100$$

Fructosamine content by NBT method

The fructosamine content was measured using a slightly modified version of the Nitro Blue Tetrazolium (NBT) Assay, which has been previously reported.[16] For the assay, NBT reagent (0.5 mM) was prepared in carbonate buffer (100 mM, pH 10.4). Now, 0.2 mL of each sample was added to 0.8 mL of NBT reagent and allowed to incubate for 15 min at 37°C. The absorbance of each tube was recorded at 530 nm, and the level of fructosamine was measured in terms of inhibition as per the given formula:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

Protein carbonyl content by 2,4Dinitrophenylhydrazine method

The analysis of protein carbonyl content was done according to a procedure described earlier.[17] In this method, 0.1 mL of each of the reaction mixtures was incubated with 0.4 mL

of 2,4-dinitrophenylhydrazine (10 mM) prepared in 2.5 M HCl for about 60 min at 37°C and then precipitated through trichloroacetic acid (20%). Next, the mixture was incubated for 5 min on ice before being centrifuged at 10,000 g for 10 min at 4°C. The resulting pellets were dissolved in guanidine hydrochloride (6 M) after being rinsed three times with 0.5 mL of a 1:1 combination of ethanol and ethyl acetate. The absorbance was taken at 370 nm, and the results were represented as % inhibition.

Fluorescence intensity of AGEs

The formation of AGEs in BSA-glucose glycation model was measured through fluorescence intensity, in which excitation occurs at 370 nm and emission occurs at 440 nm, using a spectrofluorometer.[18] The results are expressed as % inhibition as per the formula given below:

$$\% \text{ inhibition of AGE formation} = [(\text{fluorescence intensities of } A_0 - \text{fluorescence intensities of } A_1)/\text{fluorescence intensities of } A_0] \times 100$$

where A_0 = fluorescence intensities of glycated BSA; A_1= fluorescence intensities of glycated BSA treated with fractions or AG.

Assessment of glycation-induced aggregation of BSA proteins

The measurement of the aggregation index was done according to a method in which the absorbance of test samples with and without fractions was recorded at 280 nm and 340 nm.[19] The formula shown below was used to convert the results to a percentage of the aggregation index.

$$\text{Aggregation index } (\%) = A_{340} / (A_{280} A_{340}) \times 100$$

Statistical analysis

The data was presented as means SD ($n = 6$). Using the statistical programme GraphPad Prism-5.0, One-way Analysis of Variance (ANOVA) was performed on the relevant data in order to determine the quantitative differences between the experimental groups. In all tests, $p < 0.05$ was used as the criterion for statistical significance.

RESULTS

Extract preparation and yield of *P. ostreatus*

The yield of *P. ostreatus* extract using various solvent expressed on dry weight basis expressed in g/100g. In Soxhlet method, methanolic extract yield (8.38%) was maximum followed by butanol (6.35%) and aqueous (6.02%). *P. ostreatus* extract with hexane exhibited less yield (2.31%) compared to other solvent taken for study.

Quantitative analysis of TPC and TFC

The amount of Total Phenolic Compound (TPC) and Total Flavonoid Content (TFC) in methanolic, butanolic, and aqueous and hexane crude extracts *P. ostreatus* were presented in Figure 1. The obtained results were calculated from the linear regression equation of standard curve (y = 10.4x—0.8, R^2 = 0.991). The results showed that TPC of the methanolic extract (16.89 ± 1.44 mg GAE/g) is higher than that of 100% butanol (11.23 ± 0.89 mg GAE/g) and the aqueous extract (8.36 ± 0.44 mg GAE/g). Hexane extract shows the lowest phenolic content (2.34 ± 0.19 mg GAE/g). Effect of solvent on extraction of total flavonoid compound was observed similar to that on TPC. The highest TFC was obtained in the 100% methanolic extract (9.40 ± 0.86 mg QE/g) and negligible amount found in extract of *P. ostreatus* using hexane (0.83 ± 0.06 mg QE/g). The present study revealed the following manner of the solvents for the extraction of phytochemicals; Methanol>Butanol> Distilled water>Hexane.

Antioxidant study

The total antioxidant capacity of the methanolic extract was examined by the DPPH free radical scavenging ability, $ABTS^+$ free radical reducing ability and hydrogen peroxide scavenging activity assay. Methanolic extract of *P. ostreatus* was further selected for determination of antioxidant activity due to higher phenolic and flavonoid content. In the present study, the free radical scavenging ability of methanolic crude extracts of *P. ostreatus* against DPPH free radical was calculated. Figure 2 shows the DPPH scavenging activities, $ABTS^+$ free radical reducing ability and hydrogen peroxide scavenging activity assay of methanolic crude extracts of *P. ostreatus* in a concentration-dependent manner (10µg/mL-100µg/mL). Antioxidant activity found in all assay was enhanced in a concentration dependent manner. Ascorbic acid, used as the positive antioxidant control shows 100% scavenging ability. It was found that in experiment that further increase the concentration of extract shows decline in activity might be due to interference of other phytochemicals. The

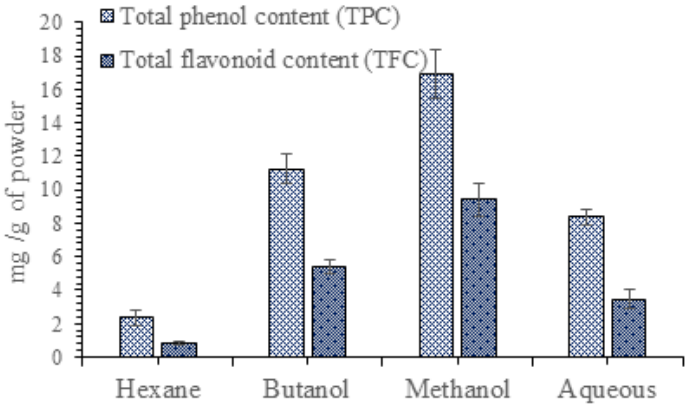

Figure 1: Total phenolic and flavonoid content of *P. ostreatus* extracted using methanol, butanol, distilled water, and hexane as solvents. The results are denoted in mean ± SD of triplet experiments.

number of antioxidants in the sample or the ability to scavenge free radicals is inversely related to the IC_{50} value. The IC_{50} values were 75.23 ± 2.32 µg/mL for DPPH radical scavenging activity, 50.33± 1.82 µg/mL for ABTS radical scavenging activity and 96.54 ± 2.32 µg/mL for hydrogen peroxide scavenging activity.

Antiglycation potential of bio-guided fraction of methanolic crude extracts of *P. ostreatus*

The powerful anti-oxidative crude methanolic extract of *P. ostreatus* were further segregated using an eluent with an increasing degree of polarity and a silica gel chromatographic column. Solvent present in each of the five fractions (F1, F2, F3, F4 and F5) was eliminated in a rotary evaporator and dissolved in appropriate amount of 1.0 mL of DMSO and investigated for glycation-reducing potential by using a fructose induced haemoglobin model and fructose-BSA model system. The antiglycation capability was determined at the initial, middle and last stage of protein glycation and compared with the standard antiglycation agent Aminoguanidine (AG). The effect of fractions F1 to F5 of *P. ostreatus* and positive control aminoguanidine on

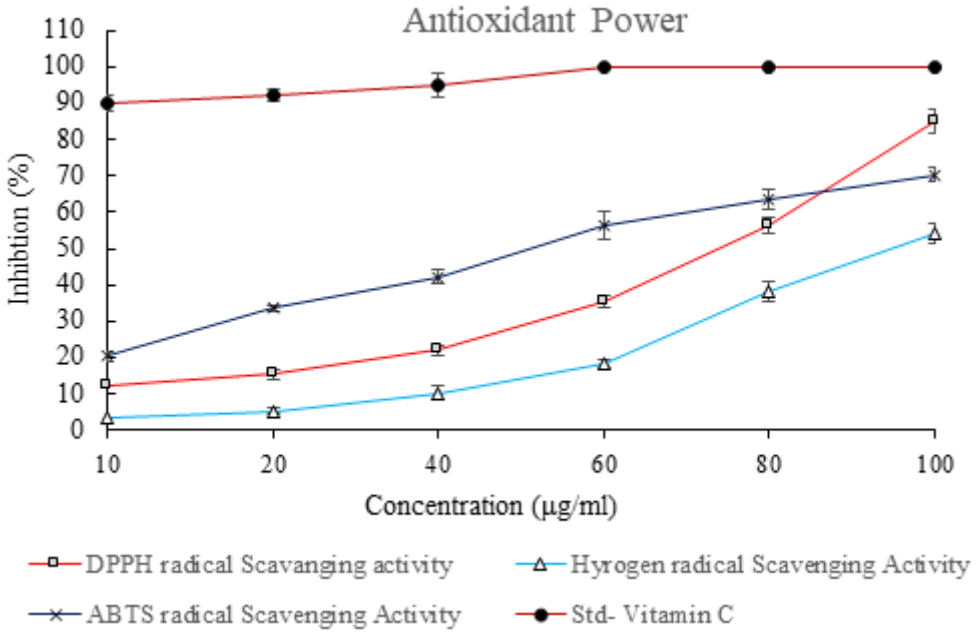

Figure 2: DPPH free radical scavenging ability, $ABTS^{+}$ free radical reducing ability and hydrogen peroxide scavenging activity of methanolic crude extracts of *P. ostreatus*.

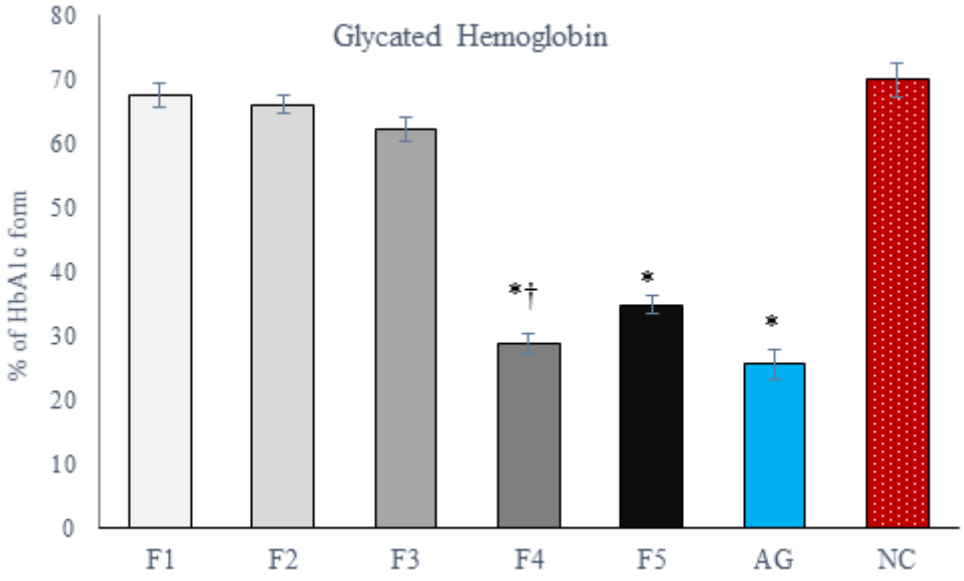

Figure 3: Inhibitory effect of fraction F1 to F5 and positive control aminoguanidine on formation of glycated hemoglobin *in vitro*. *p<0.05 when compared to negative control and † p<0.05 when compared to F5.

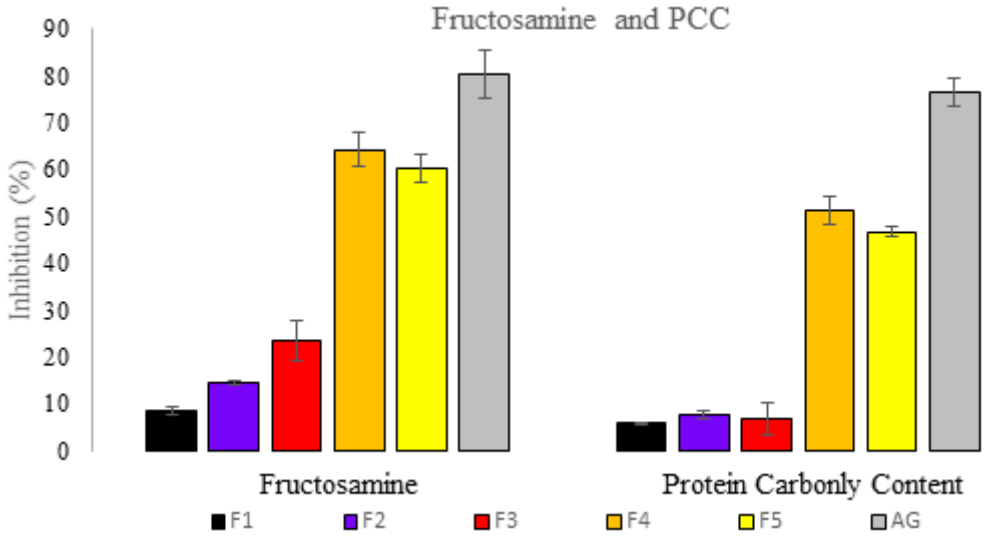

Figure 4: Determination of % inhibition of fructosamine level and the protein carbonyl content in the BSA/fructose system using five fraction of methanolic extract of *P. ostreatus.*

HbA_{1c} formation after days 28 days is shown in Figure 3. Our findings demonstrated that exposure of phytochemicals present in fraction 4 and 5 could effectively reduce the formation of glycated hemoglobin *in vitro* after 28 days of incubation. In presence of fraction F4 and fraction F5, there was significant decrease in the formation of glycated hemoglobin by 58.84% and 50.28% respectively when compared to negative control. The formation of glycated hemoglobin in the presence of fraction F1, fraction F2 and fraction F3 was not significantly differ from negative control indicates no inhibitory efficacy. The effect of five fraction obtained from crude methanolic extract of *P. ostreatus* on *in vitro* glycation of bovine albumin in the form fructosamine levels and Protein Carbonyl Content (PCC) is shown in Figure 4. At the end of the study period, fraction F4 and fraction F5 inhibited the formation of fructosamine in BSA/fructose by 64.32% and 60.24% respectively, whereas the inhibitory effect of AG as positive control was 80.34%. Protein oxidation, which takes place during the protein glycation process, was estimated using the protein carbonyl content measurement. At 28 days of incubation, fraction F4 and fraction F5 reduced the level of protein carbonyl by 51.35% and 46.7% found to be higher compared to fraction F1 to fraction F3. In addition, AG reduced the protein carbonyl content by 76.63%. The same trend of inhibition of protein carbonyl content formation was demonstrated. The glycation of protein in the middle stage leads to formation of aggregates of protein which was measured in the form of % of aggregation index and is depicted in Figure 5. The aggregation index for glycated BSA is 91.58% which is about 18 folds higher than the native BSA (5.50%). There is a significant decrease obtained by AG that showed an aggregation index up to 40.86%

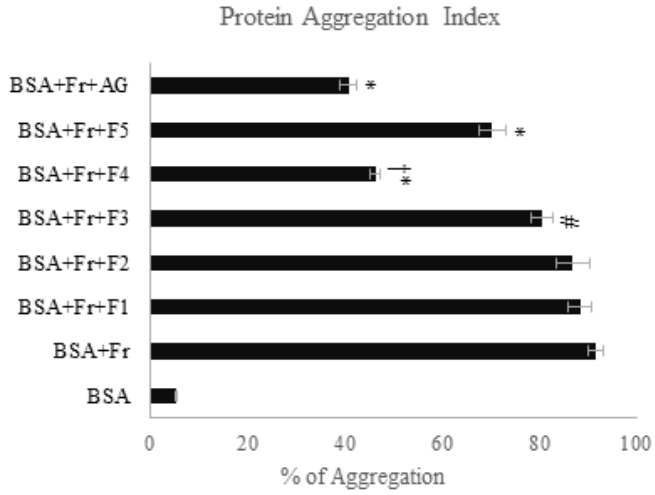

Figure 5: % Protein aggregation measured in presence of five fraction from methanolic extract of *P. ostreatus* and positive control. *p<0.001 and #p<0.05 when compared to BSA+fructose; † p<0.001 when compared to F5.

which is about 45% less in comparison with BSA-fructose. In the present study, fraction F1, fraction F2 and fraction F3 exhibited negligible protective effect on protein aggregation as indicated from aggregation index. Fructose/BSA system when treated with F4 and F5 caused a significant decrease 46.32% and 23.18% in the glycation-induced aggregation. The most significant potential for reducing protein aggregation is shown by fraction 4. To investigate the capability of fraction of methanolic extract of *P. ostreatus* in the late stage of glycation, the intensity of fluorescence was measured and indicated in Figure 6. The spectrofluorometric analysis indicates 63.27% of inhibition by AG. However, fraction F4 exhibits 52.04% of inhibition at its maximum concentration which is significantly greater among other fractions.

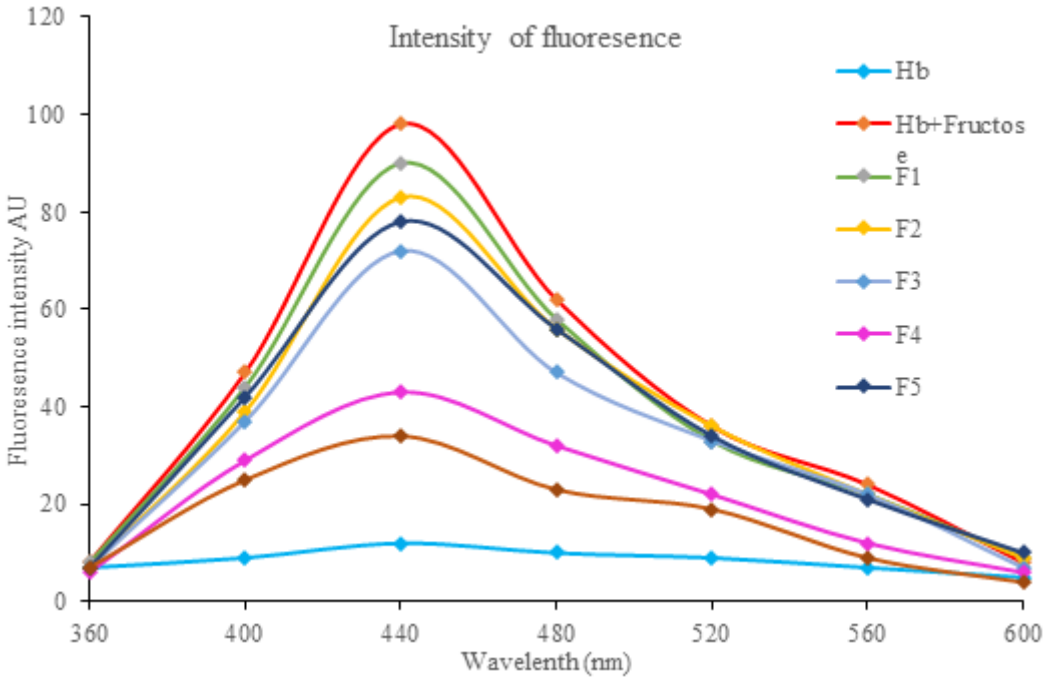

Figure 6: Analysis of the intensity of fluorescence detected from bio fractions gained from methanol extract of *P. ostreatus*.

DISCUSSION

The current investigation is mainly focused to study the protective effect of edible mushroom *P. ostreatus* on fructose induced glycation of protein haemoglobin and BSA, which leads to the occurrence of Advanced Glycated End Products (AGEs) through a series of interactions called protein glycation. Previous studies have revealed that accumulation of glycated protein in various tissue is a critical factors in the development of pathophysiological mechanisms like coronary artery disease, neurological disorders, obesity, and other diabetic complication.[20] A patient suffering from diabetes mellitus type 2 has a high rate to build-up AGEs in the tissues.[21] Glycated haemoglobin (HbA_{1c}) measurement is frequently used in the diagnosis and treatment of Diabetes Mellitus (DM) as a technique for determining long-term glycemic exposure.[22] Glycated hemoglobin also used for describing the risk of complications among diabetic patients.[23] Previously, fructose-mediated protein glycation was well studied using Bovine Serum Albumin (BSA) *in vitro*.[24] Therefore, preventing the development of AGEs is a crucial strategy for treating a variety of illnesses. The well-known AGE inhibitor Aminoguanidine interacts with the reactive carbonyls of protein to stop the accumulation of AGEs but it having number of toxic effects. Only 25 out of 2,000 types of mushrooms that can be found in nature are typically consumed in human diets. In the present study, we examine the antiglycation potential of most common edible mushroom *P. ostreatus*. Phenolics and flavonoid are thought to be an essential part of the human diet and have numerous health advantages in addition to their powerful antioxidant action.[25] In above point of view, phytochemicals were extracted using various solvent. The result of Total Phenolic (TPC) and Flavonoid Content (TFC) present in mushroom *P. ostreatus* was found with an increasing order in solvent: methanol>butanol>distilled water>hexane. Our study reported significantly higher ($p<0.05$) TPC and TFC present in methanolic extract of *P. ostreatus*. Moreover, the polarity and boiling point of methanol provides better extraction of bioactive constituents through the Soxhlet apparatus. The richness of *Pleurotus* mushrooms is not only in the nutritive aspect but also in bioactive molecules.[26] *P. ostreatus* contains major phenolic component types, including vanillic acid, myricetin, caffeic acid, homogentic acid, chrysin, routine, gentisic acid, gallic acid, protocatechuic acid, naringin, tannic acid, syringic acid, 5-O-caffeoylquinic acid, cinnamic acid, and p-coumaric acid.[27] Through a variety of biological processes, all these active substances can both prevent and treat diseases.[28] Phenolic constituents are the chief contributors to antioxidant activity. Considering this, the analysis of the anti-oxidative properties presents in the methanolic extract of *P. ostreatus* has been carried out. The current work demonstrated the extraordinary antioxidant capacity of *P. ostreatus* through its ability to scavenge DPPH radicals, hydrogen peroxide, and ABTS radicals. Shamtsyan and Pogačnik [29] reported on the antioxidant activity of *P. ostreatus* with DPPH radical-scavenging activities in the range 15–80 percent. Hydrogen peroxide can elevate oxidative stress by enhancing reactive oxygen species and causing redox imbalances. Previously, HPLC analysis revealed homogentisic acid and gallic acid present in *P. ostreatus* acts as a scavenging agent for H_2O_2.[30] According to a study,[31] mushrooms contain large levels of phenolics with higher molecular weights and

can be regarded as cationic radical scavengers. Free radicals and reactive oxygen species cause oxidative stress, which promotes the formation of AGEs and speeds up the glycation of proteins.[32] Furthermore, the initial, middle, and final stages of protein glycation are all influenced by carbonyl stress, which is another pathway that results in the production of AGEs. In this consideration, further experiments were continued with the bio-guided fractions of the methanolic extract of *P. ostreatus*, called fraction F1, fraction F2, fraction F3, fraction F4, and fraction F5, eluted using solvents in a polarity gradient. Each of the five fraction was further examined for inhibition of glycated hemoglobin, fructosamine inhibition (initial stage), protein carbonyl inhibition and protein aggregation index (middle stage), and intensity of AGE fluorescence (last stage). The result reveals that fraction F4 (ethyl acetate-methanol, 1:1) was the most effective in inhibiting formation of glycated hemoglobin and fructosamine formation when compared to other fractions. It is assumed that fraction F4 comprised the semi-polar and polar phenolic and flavonoid such as flavonoids (i.e., aglycon and glycosides), phenol hydroquinones, alkaloids, and some sterols, are extracted in ethyl acetate reported previously.[33] The formation of protein carbonyls is one of the indicators of glycation-induced protein oxidation (glycoxidation), which is greatly inhibited by fraction F4 and fraction F5. It is assumed that this fraction contains certain components that can trap the dicarbonyls and hence reduce the protein carbonyl content. In a further progression of glycation, aggregates of proteins are produced, which are significantly reduced in fraction F4-treated glycated BSA. The reason behind the decline in the aggregation index could be phenolic acids and flavonoids with a lower molecular weight that have anti-amyloidogenic effects, which make them effective in halting the formation of amyloids.[34] The study of *P. ostreatus* by Gąsecka *et al*.[35] were detected ferulic, p-coumaric, 4-hydroxybenzoic, protocatechuic, t-cinnamic, vanillic acids and myricetin. Recently, the antiglycation function of p-coumaric,[36] ferulic acid,[37] protocatechuic,[38] t-cinnamic,[39] Vanillic acid[40] has been well reported. According to the spectrofluorometric analysis of the BSA-fructose model, fraction F4 shows most significantly decline in the intensity of fluorescence suggest effectively inhibited the fluorescent AGE formation. Overall fraction F4 from methanolic extract of *P. ostreatus* display excellent antiglycation activity compared to other fraction on the multiple stages of protein glycation which need to be further invested for its constituents.

CONCLUSION

The methanolic extract of *P. ostreatus* has significant anti-oxidative potential due to the overall phenolic and flavonoid content. The current study demonstrated the remarkable potential of the fraction F4 from the methanolic extract of *P. ostreatus* on fructose-mediated haemoglobin as well as BSA protein glycation at three different stages: early, when glycated hemoglobin and fructosamine was inhibited, middle, when protein carbonyl content was inhibited and protein aggregation index was decreased, and late, when fluorescent AGEs were reduced. The results of this investigation thus imply that fraction F4 obtained from the methanolic extract of *P. ostreatus* can be employed for the discovery of bioactive chemicals that can then be taken into consideration as therapeutic agents for AGEs-associated pathophysiology.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AGE: Advanced Glycation End Product; **GAE:** Gallic acid equivalent; **ABTS:** 2,2'-Azinobis-(3-ethylbenzothiaz oline-6-sulfonate; **DPPH:** DPPH (2,2-diphenyl-1-picrylhydrazyl); **QE:** Quercetin equivalent; **TPC:** Total phenolic content; **TFC:** Total flavonoid content; **BSA:** Bovine serum albumin; **HbA_{1c}:** Glycated hemoglobin; **NBT:** Nitro Blue Tetrazolium.

SUMMARY

Hyperglycemia significantly contributes to the development of the long-term complications of diabetes and aging via increased accumulation of protein glycation in tissues. Carbonyl stress is an additional contributor to cell damage that has been associated with a number of human disorders. The current study investigates antioxidant activity and anti-glycation capacity of methanolic extract of *P. ostreatus*. The methanolic extract of *P. ostreatus* display excellent antioxidant activity due to phenolic rich extract. The most active fractions were chosen bio-guided based on their antiglycation capabilities. Fraction F4 of *P. ostreatus* extract is the most potent in inhibiting HbA_{1c}, fructosamine, and protein carbonyl production. *P. ostreatus* could be a natural source for a potential nutraceutical resource for reducing carbonyl stress and AGEs-related diseases.

AUTHORS'S CONTRIBUTION

DM has carried out the all the antioxidant and antiglycation analysis along with all the other experiments and manuscript drafting. HVP has carried out the work design as well as editing of the manuscript.

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