Inhibitory effect of *Piper betle* Linn. leaf extract on protein glycation – Quantification and characterization of the antiglycation components

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*Piper betle* Linn. is a Pan-Asiatic plant having several beneficial properties. Protein glycation and advanced glycation end products (AGEs) formation are associated with different pathophysiological conditions, including diabetes mellitus. Our study aims to find the effect of methanolic extract of *P. betle* leaves on *in vitro* protein glycation in bovine serum albumin (BSA)-glucose model. The extract inhibits glucose-induced glycation, thiol group modification and carbonyl formation in BSA in dose-dependent manner. It inhibits different stages of protein glycation, as demonstrated by using glycation models: hemoglobin-δ-glucosone (for early stage, Amadori product formation), BSA-methylglyoxal (for middle stage, formation of oxidative cleavage products) and BSA-glucose (for last stage, formation of AGEs) systems. Several phenolic compounds are isolated from the extract. Considering their relative amounts present in the extract, rutin appears to be the most active antiglycating agent. The extract of *P. betle* leaf may thus have beneficial effect in preventing protein glycation and associated complications in pathological conditions.

**Keywords:** *Piper betle*; Protein glycation, Advanced glycation end products, Diabetes mellitus, Rutin

Protein modification by non-enzymatic glycation is a complex series of reactions, collectively called the Maillard reaction, which may be significant with increased level of blood sugar (hyperglycemia) over prolonged periods of time. Glucose reacts with protein amino groups to form labile Schiff base that rearranges to an early glycation (Amadori) product. This product undergoes further reactions involving reactive dicarbonyl intermediates such as 3-deoxyglucosone and methylglyoxal to form complex, heterogeneous, fluorescent and cross-linked structures, called advanced glycation end products (AGEs). AGEs are associated with increased formation of free radicals, causing oxidative stress and associated complications. The primary objective of all diabetic treatment is, therefore, to maintain blood glucose level within the normal range. Several synthetic AGE inhibitors have been tried in the treatment of diabetes without much success, due to their relatively low efficacies, poor pharmacokinetics and toxicities.

In recent years, major research has been directed to find appropriate antihyperglycemic or antiglycating agents from plant origins. In an *in vitro* study, Wu and Yen have used different assay systems to investigate the effect of several flavonoids on different stages (early, middle and late) of protein glycation. The inhibitory mechanism of flavonoids against glycation has been attributed, at least partly, due to their antioxidant properties. Lee et al have shown that ethylacetate extract of the fruiting body of a medicinal mushroom *Phellinus linteus* possesses inhibitory effect against protein glycation. They have identified several compounds, which inhibit different stages of glycation to different extents. These findings are quite encouraging exploring antiglycating agents from different plant sources.

*Piper betle* Linn. is an important plant in the Asiatic region. It is cultivated in India, Srilanka, Malay, Peninsula, Philippine Islands and East Africa. Kumar et al have discussed in detail the pharmacological activities of *P. betle*. Although it has many medicinal properties, such as stimulative, carminative, aphrodisiac, gastroprotective, hepatoprotective, and antioxidant, its leaves are generally consumed daily by nearly 600 million people not as a medicine, but as a habit or
addiction. The traditional use involves chewing of its leaves and ranks second to coffee and tea in terms of daily consumption.

*P. betle* leaf is not used as an antidiabetic agent in traditional medicinal systems, but its antihyperglycemic activity has been reported in streptozotocin (STZ)-induced diabetic rats[^6^,^7^]. However, its antiglycation activity has not yet been explored. We have, therefore, undertaken an *in vitro* study to demonstrate the effect of methanolic extract of *P. betle* leaf on protein glycation. The extract inhibits all stages (early, middle and late) of protein glycation. Several phenolic compounds are present in the extract, in which rutin appears to be the most active against protein glycation.

### Material and Methods

#### Materials

Bovine serum albumin (BSA, essentially fatty acid free), D-glucose, nitroblue tetrazolium (NBT), 2,4-dinitrophenylhydrazine (DNPH), 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), δ-gluconolactone, methylglyoxal, acrylamide, bisacrylamide, rutin, catechin, epicatechin, protocatechuic acid, ferulic acid and chlorogenic acid were purchased from Sigma Chemical Co., USA. HPLC grade methanol, acetonitrile and acetic acid were purchased from Spectrochem, India. Glucose and glycohemoglobin kits were obtained from Span Diagnostics, India and Eagle Diagnostics, USA, respectively. Other chemicals were of analytical grade and purchased from Sisco Research Laboratory, India.

#### Plant material extraction

*Piper betle* (Bangla variety) leaves were collected from local markets. Their herbarium specimens were prepared and taxonomically identified according to the classification method of Prain[^8^]. The leaves were detached and dried in oven at 60°C for 1 h. Dry leaves (25 g) were crushed in a mortar-pestle with methanol. Water and chloroform-soluble fractions were separated step-wise from the methanolic fraction by using a silica column (10 x 1 cm). The resulting fraction was sonicated for 1 h under cold condition and filtered through a Whatmann-I filter paper. The filtrate was lyophilized and the fine powder (14 mg) thus obtained was used to make a methanolic stock solution (1 mg/ml) (Fraction A) and was used in the present study. The extraction procedure is shown in **Scheme 1**.

#### In vitro non-enzymatic protein glycation

BSA-glucose model system was used to evaluate the antiglycation activity of the fraction A. BSA (20 mg/ml) was mixed with glucose (500 mM) in 0.2 M potassium phosphate buffer, pH 7.4 in the presence of sodium azide (0.02 %). Different amounts of fraction A (0, 6, 30, 60, 90, 120 and 150 µg) were added to the reaction mixture (1 ml) and incubated at 37°C for 21 days. For control experiment, only BSA was incubated under identical conditions. After incubation, the reaction mixtures were used in the following experiments:

#### Biochemical estimations

Amount of free glucose in the incubated samples was measured by using glucose estimation kit following the conventional glucose oxidase-peroxidase (GOD-POD) method[^9^]. Free glucose in the reaction mixtures gave an estimate of the amount of glucose consumed and extent of glycation of the protein. Fructosamine content in the incubated samples was measured by using NBT according to the method of Baker *et al[^10^]*. The amount of carbonyl content formed in the incubated BSA samples was measured by reaction with DNPH following the method of Levine *et al[^11^]*. Thiol groups in the incubated BSA samples were measured according to the method of Ellaman[^12^] using DTNB.

#### Spectroscopic studies

The incubated samples were diluted to 6 µM BSA for all spectroscopic studies. The samples were subjected to absorption spectral analysis (250-350 nm) by using UV/VIS Spectrophotometer (Hitachi U2000). The fluorescence emission spectra of the samples were monitored with excitation at 285 nm in a Spectrofluorimeter (Hitachi F-3010). The CD
spectra (200-250 nm) of the samples were recorded after averaging nine scans for each sample in a Spectropolarimeter (Jasco 600) using 1 mm path length cuvette and α-helical contents were determined according to the method of Geraci and Parkhurst\(^\text{13}\).

**Experiments on the effect of fraction A on different stages of glycation**

**Early stage of glycation**

Early stage of glycation (Amadori product formation) was evaluated by using hemoglobin-δ gluconolactone system according to the method of Rahbar and Nadler\(^\text{14}\). Fresh human blood (200 µl) from normal healthy volunteers was incubated with 40 µl δ-gluconolactone (50 mM) in phosphate buffer saline (PBS), pH 7.4 (total volume 1 ml) in the absence or presence of different amounts of fraction A at 37°C for 16 h. After incubation, percentage of glycated hemoglobin was determined by using glycohemoglobin kit.

**Middle stage of glycation**

BSA-methylglyoxal system was used to study the middle stage of glycation following the method of Wu and Yen\(^\text{3}\). The reaction mixture (1 ml) containing BSA (50 mg) and methylglyoxal (100 mM) in 0.1 M phosphate buffer, pH 7.4 was incubated aseptically in the absence or presence of different amounts of fraction A at 37°C for 9 days. After incubation, the mixtures were subjected to SDS-PAGE (10%) to find the effect of fraction A on methylglyoxal-induced glycation of BSA.

**Late stage of glycation**

Late stage of glycation was studied by using BSA-glucose system. The reaction mixture (1 ml) containing BSA (50 mg) and glucose (500 mM) was incubated at 37°C for 21 days in the absence or presence of different amounts of fraction A. The fluorescence emission of the reaction mixtures was then monitored at 450 nm with excitation at 370 nm, as a characteristic of AGE formation\(^\text{3}\). The mixtures were also subjected to native-PAGE (10 %) to find the effect of fraction A on cross-linked AGE formation in BSA\(^\text{15}\).

**Characterization of phenolics in fraction A and estimation of their antiglycation activities**

For characterization of phenolics in fraction A, the fraction was subjected to HPLC (LC-10AT VP Liquid chromatograph, Shimadzu) analysis. HPLC photodiode array detector was set at 280 nm or 370 nm for detection of phenolics. C-18 column [Luna 5u C18 (2) 100A, size-250 x 4.60 mm, Phenomenex, USA] was used for HPLC separation. Water/methanol/acetonitrile/acetic acid (89:9:1:1) mixture was used as mobile phase (flow rate 0.5 ml/min and scan time 100 min)\(^\text{16}\). The antiglycation activities of different phenolics isolated from fraction A were estimated from glucose consumption in BSA-glucose system as described in earlier section.

**Statistical analysis**

The results were expressed as mean ± SEM. The significance was determined by using unpaired two-tailed student’s t test and one way analysis of variance (ANOVA). The level of significance was established at P< 0.05.

**Result and Discussion**

Water and alcohol soluble fractions of \textit{P. betle} leaves have been reported to exhibit antidiabetic effect in STZ-induced diabetic rats\(^\text{7}\). Santhakumari \textit{et al}\(^\text{6}\) have also reported the antihyperglycemic effect of \textit{P. betle} leaf in STZ-diabetic rats. Several compounds namely, chevibetol, allylpyrocatechol and their respective glucosides have been isolated from ethanolic extracts of different varieties of \textit{P. betle}\(^\text{17}\). These constituents exhibit antioxidant activities. Although the mechanism of antihyperglycemic action of \textit{P. betle} leaf is not yet clear, the antioxidants present in the leaves may contribute, at least partly, in this respect. Here we have studied in detail the \textit{in vitro} effect of the methanolic extract (fraction A) of \textit{P. betle} (Bangla variety) leaves on protein glycation.

**Effect of fraction A on glycation of BSA: Biochemical changes**

The reaction mixtures containing BSA and glucose (BG) were incubated at 37°C for 21 days in the absence and presence of different concentrations of fraction A and glycation-induced biochemical changes in the protein were studied (Table 1).

The extent of glycation was estimated from the amount of glucose consumed by BSA, for which free glucose remained in the incubated mixtures was measured. Fraction A exhibited an inhibitory effect on glycation of BSA, as revealed by gradually reduced glucose consumption by BSA in the presence of increased amount of the fraction. However, even highest amount of the fraction used in the study (150 µg) was not able to completely prevent glycation of the protein.
Fructosamine is the early product (Amadori product) of protein glycation, and it undergoes oxidative cleavage, resulting in the formation of AGEs. Fructosamine formation due to glycation was measured in the incubated mixtures. Glycation enhanced fructosamine formation (82.53 µmol/mg BSA), which was gradually reduced by increased concentration of fraction A, as shown in Table 1.

Glycation-induced covalent modification of proteins by introducing carbonyl groups into amino acid residues of proteins is well-known\(^\text{18}\). In carbonyl stress, oxidatively modified proteins are susceptible to degradation. Carbonyl formation was significantly increased by glycation (13.99 µmol/mg BSA) in comparison with the control having no glucose (4.31 µmol/mg BSA). Fraction A was quite effective in inhibiting the modification caused by glucose (Table 1).

Glycation-induced thiol group modification is known in diabetic condition\(^\text{19}\). Glycation decreased the content of free thiol groups in BSA (65.02 nmol/mg BSA to 21.34 nmol/mg BSA), indicating their modifications. However, fraction A prevented the modification, resulting in gradual increase in free thiol groups in the protein. The extract (150 µg) increased thiol group content in BSA from 21.34 nmol to 47.54 nmol/mg protein (Table 1).

The dose-dependent inhibitory effect of fraction A on the glycation parameters namely, fructosamine formation, carbonyl content and thiol group modification in BSA might be directly associated with lowering of the extent of glycation.

### Effect of fraction A on glycation of BSA: Structural modifications

The structural modifications in BSA due to glycation and the effect of fraction A on such modifications were studied by different spectroscopic methods (Fig. 1). The absorption spectra in the region 250-350 nm (Fig. 1A) showed that after 21 days incubation, the reaction mixture (BG) exhibited much higher absorbance around 280 nm in comparison with only BSA. However, the increase in absorbance...
around 280 nm reduced gradually in the presence of increased concentration of fraction A in the BG reaction mixture, indicating effective prevention of structural modification by the extract.

When excited at 285 nm, BSA exhibited emission maximum at 340 nm, as shown in Fig. 1B. The intrinsic fluorescence of the protein after glycation was significantly higher, and might be related with increased absorbance of the modified protein around 280 nm. However, in absence of shift in emission maxima, it is difficult to suggest higher solvent accessibility of tryptophan residues of the glycated protein. The protein fluorescence appeared to reduce gradually in the presence of increased amount of fraction A in the BG reaction mixture.

Figure 1C shows the CD spectra of the proteins (control BSA and BG mixture in the absence or presence of fraction A) at far UV region (200-260 nm). Compared to BSA, BG mixture after incubation exhibited significantly reduced negative ellipticity in the region 210-225 nm. Molar ellipticity \([\theta]\) values were obtained using the relation \([\theta] = [M,W]/10,l,c\), where \(c\) (g/ml) is the concentration of protein, \(\theta\) (mdeg) is the observed rotation, \(l\) (cm) is the pathlength, and \([M,W]\) is the mean residual molecular weight of the amino acid \(([M,W] = 110)\). The \(\alpha\) helical contents of the protein samples were estimated according to the relation: Fraction of \(\alpha\)-helix = \(([\theta]_{222} + 2340) / 30300\), where \([\theta]_{222}\) is the ellipticity at 222 nm. The \(\alpha\)-helical contents of BSA and BG mixture appeared to be approximately 71% and 46%, respectively. Glycation thus reduced the level of \(\alpha\)-helix (approximately 25%) in BSA, leading to an increase in volume of the protein. Addition of fraction A in the BG mixture during incubation resulted in increased negative ellipticity as well as increased \(\alpha\)-helical content, indicating protective effect of the extract against modification of the secondary structure of the protein.

Increased fluorescence and reduced \(\alpha\)-helix content of the glycated BSA were in agreement with our previous findings on glucose and fructose modified heme proteins hemoglobin\(^{20,21}\) and myoglobin\(^{22,23}\). Fraction A prevented glycation and associated conformational changes in BSA, as revealed by gradual reversal of the structural changes in the presence of increased concentration of the extract in the reaction mixture.

**Effect of fraction A on different stages of glycation**

To understand further the inhibitory effect of *P. betle* leaf extract on protein glycation, different stages of glycation were studied using different models (Fig. 2). Currently, no suitable glycation system is known for testing inhibition at different stages of glycation by a particular inhibitor. For this purpose, different protein glycation systems mimicking different stages of glycation are used.

Hemoglobin-\(\delta\)-gluconolactone system is used to identify the early stage (Amadori product formation) of glycation\(^{24}\). \(\delta\)-Gluconolactone, an oxidized (ketoneehydride) analogue of glucose reacts with hemoglobin to form an Amadori product HbA\(_{1c}\). In
In our study, early stage of glycation or Amadori product formation was measured by using glycohemoglobin kit in hemoglobin-δ-glucolactone system in the absence or presence of different amounts of fraction A and is shown in Fig. 2A. After incubation, the content of glycated hemoglobin was significantly higher in hemoglobin-δ-glucolactone reaction mixture in comparison with the control set containing only hemoglobin (fresh blood). However, glycated hemoglobin level decreased gradually in the presence of increased amount of fraction A in hemoglobin-δ-glucolactone reaction mixture.

In the middle stage of protein glycation, Amadori products form reactive carbonyl compounds, namely methylglyoxal, glyoxal etc, which, in turn, react with proteins. Reaction of a dicarbonyl compound with proteins thus mimics middle stage of protein glycation. BSA-methylglyoxal system is in use for studying inhibition of middle stage of glycation.

In our study also, BSA and methylglyoxal reaction mixture was used for middle stage glycation assay. After incubation in the absence or presence of different amounts of fraction A, the reaction mixtures were subjected to SDS-PAGE and the protein band intensities, measured by using Quantity One software (Bio-Rad) are shown in Fig. 2B. In comparison with the control set containing only BSA (lane 1), BSA-methylglyoxal incubation mixture exhibited significantly reduced protein band intensity (lane 2), indicating glycation-induced oxidative cleavage of the protein. As the concentration of fraction A was increased gradually in BSA-methylglyoxal reaction mixture, the proteolytic cleavage appeared to be progressively inhibited (lanes 3 to 8).

In the late stage of protein glycation, AGE formation and/or cross-linking of proteins occur. Long-term (21 days) incubation of BSA-glucose mixture was used to find the effect of *P. betle* leaf extract on the late stage of glycation. AGE formation was detected by fluorescence emission of the protein at 450 nm with excitation at 370 nm (Fig. 2C). The amount of AGE formed by glycation of BSA was quite high and was prevented gradually by addition of increased amounts of fraction A in the reaction mixture. In native-PAGE (Fig. 2D), BG reaction mixture exhibited a high molecular weight protein band, indicating an AGE-induced cross-linked structure formed in the process (lane 2), in comparison with that in the control set (lane 1). When BG was incubated with fraction A, the high molecular weight protein band gradually disappeared (lanes 3 to 8), suggesting the inhibitory effect of the extract on AGE formation.

Protein glycation leads to AGE accumulation, which, in turn, prevents normal function of the cells. Intervention of AGE formation is, therefore, an important approach in preventing diabetic complications. Santhakumari *et al.* and Arambewela *et al.* have reported antihyperglycemic activity of *P. betle* leaf extract. Our findings suggested that fraction A possesses strong antiglycation activity by inhibiting all stages of protein glycation. Flavonoids have been reported to inhibit different stages of protein glycation. The ethylacetate extract of the fruiting body of the mushroom *Phellinus linteus* has also been shown to possess an inhibitory effect against protein glycation.

**Characterization of phenolics in fraction A**

Major phenolic compounds (polyphenols and flavonoids) present in fraction A were determined by HPLC. The presence of epicatechin, catechin, rutin, protocatechuic acid, ferulic acid/chlorogenic acid and gallic acid in fraction A was confirmed by HPLC. C-18 column was used with water/methanol/acetonitrile/acetic acid (89: 9: 1: 1) as the mobile phase. The separated compounds were identified by comparing the chromatograms (retention time) of the standard phenolic compounds subjected to HPLC under similar condition; and FT-IR spectra of rutin sample separated from fraction A by HPLC and known standard rutin.
comparison of the retention time of the eluted fractions as well as standards treated under similar conditions. The elution of the components was monitored by absorbance recorded at 280 nm (Fig. 3A). All these compounds, except rutin had absorption maxima at 280 nm. Rutin was confirmed by its absorption maximum at 370 nm (not shown), as well as FT-IR spectrum together with the standard (Fig. 3B). From HPLC analysis, the amounts of individual phenolic compounds in fraction A (150 µg) were found to be epicatechin (33.45 µg), rutin (24.37 µg), catechin (21.61 µg), gallic acid (16.54 µg) and protocatechuic acid (14.85 µg). Standard ferulic acid and chlorogenic acid showed same retention time (63 min) in HPLC. A component was also separated from fraction A at the same position of ferulic acid/chlorogenic acid. However, this component could not be identified.

The antiglycation activities of different phenolic compounds present in fraction A were estimated from inhibition of in vitro glycation of BSA. The antiglycation efficiencies with respect to the relative amounts of different components present in 150 µg fraction A are shown in Table 2. Fraction A (150 µg) inhibited about 55% glycation, while rutin (24.37 µg) present in the same amount of the fraction was effective in inhibiting about 18.67% glycation, which appeared to be the maximum among the individual components. Antiglycation activities per 100 µg of different phenolics present in fraction A were also included in Table 2. Synergistic effect of different components may play important role in the overall antiglycation activity of fraction A and needs further study.

Fraction A exhibited strong antioxidant activity (not shown), which might be due to its phenolic compounds. Fraction A may exert antiglycation activity through the antioxidant properties of its phenolic compounds, particularly rutin, which may interrupt the cascade of events, causing prevention of glycation. However, further studies are necessary to understand, if the antiglycation activity of the extract is related to its free radical scavenging activity.

In conclusion, P. betle leaf extract possesses strong antiglycation activity and may be quite useful in the treatment of pathological complications including diabetes mellitus.

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