Research Article

In Vitro α-Amylase and Protein Glycation Inhibitory Activity of the Aqueous Extract of Flueggea leucopyrus Willd

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There is much interest in plant-based medicine with antidiabetic and antiglycation properties. Chronic hyperglycemia plays a vital role in the development of long-term diabetic complications by inducing protein glycation and the gradual formation of advanced glycation end products (AGEs) in various body tissues. The main objectives of this study were to investigate the aqueous extract of the whole plant of Flueggea leucopyrus Willd (FLAE), a medicinal plant used in traditional medicine in Sri Lanka for its in vitro α-amylase inhibitory activity and its inhibitory potential on the formation of AGEs. α-Amylase inhibitory activity determined by 3,5-dinitrosalicylic acid method revealed that FLAE possesses 29%–91% inhibitory activity at a concentration range of 2.5–400 μg/mL, respectively. Nonenzymatic protein glycation inhibitory capacity assessed by bovine serum albumin-fructose fluorescence spectrometric assay showed that FLAE at 15.6–250.0 μg/mL inhibited AGE formation by 0.9%–98%, respectively. Radical scavenging ability of FLAE using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and total phenolic and flavonoid contents of FLAE were also determined. This study shows that Flueggea leucopyrus Willd not only inhibits α-amylase enzyme, which is known to break starch to glucose, but also inhibits the formation of AGES, which occur due to chronic hyperglycemia that leads to the onset of diabetic complications.

1. Introduction

In spite of the advances in modern medicine, plant-based remedies are being searched for treatment of many diseases, including diabetes mellitus and associated complications due to the formation of advanced glycation end products (AGEs) [1–3]. Postprandial hyperglycemia is an important initial characteristic of diabetes mellitus, and the reduction in postprandial glycemic levels is one of the major concerns in the treatment of diabetes mellitus [4]. Inhibiting the enzyme α-amylase has become one of the most common targets for decreasing postprandial hyperglycemia [5]. α-Amylase is a key carbohydrate hydrolyzing enzyme in the gastrointestinal tract, which breaks α-1,4-glycosidic bonds in polysaccharides and releases glucose to the blood stream. Hence, the inhibition of this enzyme would prolong the degradation of starch and oligosaccharides to glucose in the intestines and thus control the glycemic rise in the post-prandial stage [5–8]. Recently, a vast number of research studies have been carried out to find potential natural antidiabetic agents since currently used drugs such as acarbose result in noticeable gastrointestinal side effects and toxicity [7, 8]. Plant-based remedies that have fewer side effects in comparison to synthetic drugs are being searched for the treatment of diabetes mellitus. Therefore, plant extracts are extensively screened in search of α-amylase inhibitors [9].

Chronic hyperglycemia plays a vital role in the development of long-term diabetic complications by inducing protein glycation and the gradual formation of advanced glycation end products (AGEs) in various body tissues [2, 5, 10]. Formation of AGEs happens in normal physiological conditions, but it is accelerated in the presence of
2. Experimental Procedures

Bovine serum albumin (BSA), aminoguanidine hydrochloride, α-amylase from Aspergillus oryzae, 3,5-dinitrosalicylic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma Aldrich USA. D-Fructose, corn starch, sodium-potassium tartrate tetrahydrate, quercetin, and sodium nitrite were purchased from HiMedia Laboratories, India. Potassium dihydrogen orthophosphate was procured from Qualigens Fine Chemicals, India. Sodium phosphate dibasic dihydrate and Folin–Ciocalteu’s reagent were purchased from Loba Chemie Pvt. Ltd., Mumbai. Sodium hydroxide pellets and anhydrous sodium carbonate were procured from Sisco Research Laboratories, and gallic acid and butylated hydroxytoluene were from Research-Lab Fine Chem Industries, India. Sodium azide and aluminium chloride were purchased from Central Drug House Pvt. Ltd., Mumbai and Fluka Chemical Corp., USA, respectively.

Acarbose from GlucoBay (50 mg) brand, Bayer Schering Pharma, was purchased from the market.

2.1. Preparation of the Plant Extract. Flueggea leucopyrus Willd plant materials were collected from the premises of Nawinna Raja Maha Viharaya (GPS coordinates 6.8817963, 79.9243362), and the identification and verification of the plant was carried out at Bandaranayake Memorial Ayurvedic Research Institute at Nawinna, Sri Lanka, and voucher specimen was deposited at same premises (accession no. 117 (a)). All parts of the collected twig were washed thoroughly with running water, air dried until it acquired a constant weight, and milled into fine particles. These fine plant parts were extracted with water according to the method of preparation of decoctions (“kasaya”) in Ayurvedic medicine explained by Perera et al. [23]. From the powdered sample, 60 g was simmerly boiled with 960 mL of distilled water until the volume of the extract was reduced to about 240 mL. Then, the extract was cooled down to room temperature and filtered through a cheesecloth. The aqueous extract was lyophilized using a freeze dryer LABCONCO FreeZone 2.5 (model no: 7670530). The powder extract was stored in 0–4°C until further use. This powder was reconstituted with deionized water/buffer to prepare required concentrations of the extract and used for all experiments.

2.2. In Vitro α-Amylase Inhibitory Activity. Inhibition of α-amylase activity by the aqueous extract of Flueggea leucopyrus Willd was carried out according to a previously published methods by Poongunaran et al. [24] and Kamtekar et al. [25] with slight modifications. α-Amylase (5 U/mL, 1.0 mL) was mixed with plant extract (0.025–4.0 mg/mL, 1.0 mL) and phosphate buffer (20 mM, pH 6.9, 1.0 mL) and incubated at 32°C for 10 minutes. Corn starch (1% w/v in 20 mM phosphate buffer, pH 6.9) 1.0 mL was added to the reaction mixture and incubated again for 10 minutes at 32°C. After adding 1.0 mL of DNS reagent (96 mM 3,5- dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2N NaOH) to each reaction mixture tubes were incubated at 85°C for 5 minutes. After incubation, reaction mixtures were cooled down to room temperature, and volume of each tube was brought up to 10.0 mL with deionized water, and the absorbance of the orange color was measured at 540 nm using UV/Vis spectrometer (Hitachi-Japan U 2910). Control was prepared without the extract, whereas the control blank did not contain both the extract and the enzyme. Test blanks were prepared with each concentration of the extract without the enzyme. The percentage inhibition (%) of α-amylase was determined as follows:

\[
\text{Inhibition} \% = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100
\]
where \( A_C \) is the absorbance of the control, \( A_{CB} \) is the absorbance of the control blank, \( A_T \) is the absorbance of the test, and \( A_{TB} \) is the absorbance of the test blank. Acarbose (10–400 \( \mu \)g/mL) a commercially available \( \alpha \)-amylase inhibitory drug was used as the positive control.

### 2.3. In Vitro Protein Glycation Inhibition

In vitro inhibitory activity of fructose induced protein glycation was determined according to a previously published method by McPherson in 1988 [26] and Fernando et al. [27] with slight modifications. Briefly, fructose (1000 mM, in 200 mM phosphate buffer pH 7.4) 4.0 mL was incubated with 5.0 mL of BSA (20 mg/mL, in 200 mM phosphate buffer, pH 7.4), 1.0 mL of FLAE (final concentration: 15.6–500 \( \mu \)g/mL), and 10 mL of phosphate buffer (200 mM, pH 7.4) at room temperature for one week. A control was prepared using only BSA and fructose in order to induce the formation of the AGEs and to compare the inhibitory activity of the extract. Control blank was prepared using only BSA, whereas sample blanks were prepared only with the plant extract with respective concentrations. The total volume of the tubes was brought up to 20.0 mL with buffer. The fluorescence emission of each mixture was measured with the excitation and emission wavelengths at 355 nm and 440 nm, respectively, using fluorescence spectrometer (Hitachi-Japan F-2700). The percentage inhibition of fluorescent AGE formation (I%) was calculated using the following equation:

\[
I\% = \frac{\left( F_C - F_{CB} \right) - \left( F_S - F_{SB} \right)}{\left( F_C - F_{CB} \right)} \times 100%,
\]

where \( F_C \) is the fluorescence intensity of the control, \( F_{CB} \) is the fluorescence intensity of the control blank, \( F_S \) is the fluorescence intensity of the sample, and \( F_{SB} \) is the fluorescence intensity of the sample blank. Aminoguanidine (1.25, 0.75 and 0.25 mg/mL) was used as the positive control.

### 2.4. Determination of the Free-Radical Scavenging Activity

The ability to scavenge the stable radical DPPH by the antioxidants present in the aqueous extract of *Flueggea leucopryrus* Willd was carried out according to a method discussed by Kuganesan et al. [28]. The test samples (25–200 \( \mu \)g/mL) were mixed with DPPH (0.05 mg/mL, 2.0 mL) and methanol (95%, 0.5 mL) and allowed to stand for 10 minutes at room temperature. Absorbance of the samples was measured at 517 nm using UV/Vis spectrophotometer (Hitachi-Japan U 2910). DPPH with methanol was used as the control. Percentage free radical scavenging activity (I%) was calculated as follows:

\[
I\% = 1 - \left( \frac{A_S}{A_C} \right) \times 100%,
\]

where \( A_S \) is the absorbance of the sample and \( A_C \) is the absorbance of the control. Butylated hydroxy toluene (BHT) (25–200 \( \mu \)g/mL) was used as the positive control.

### 2.5. Determination of the Total Phenolic Content

Total phenolic content (TPC) of the aqueous extract of *Flueggea leucopryrus* Willd was determined by Folin–Ciocalteu (FC) method according to Kamtekar et al. [25]. Different concentrations of gallic acid (20–90 \( \mu \)g/mL) were used to plot the standard curve. Each gallic acid concentration (0.5 mL) was mixed with the FC reagent (0.5 mL) and allowed to stand for 5 minutes at room temperature. \( \text{Na}_2\text{CO}_3 \) solution (6% w/v, 0.5 mL) was added to the samples followed by deionized water (2.0 mL). The tubes were incubated in the dark for 60 minutes to develop a dark blue color, and the absorbance was recorded at 765 nm using UV/Vis spectrometer (Hitachi-Japan U 2910). The blank was prepared using FC reagent with deionized water. The extract was prepared in the same manner, and the absorbance was recorded. Using the standard curve, the milligrams of gallic acid equivalents per dry weight of the extract (GAE/g) was obtained.

### 2.6. Determination of the Total Flavonoid Content

Total flavonoid content (TFC) was determined using aluminium chloride colorimetric assay [26]. Quercetin was used to plot the standard curve. To each concentration of the quercetin (0.5 mL), deionized water (2.0 mL) was added followed by \( \text{NaNO}_2 \) (5% w/v, 0.15 mL). Reaction mixtures were incubated at room temperature for 5 minutes. Then \( \text{AlCl}_3 \) solution (10% w/v, 0.15 mL) was added to each mixture, mixed well, and allowed to stand for 6 minutes at room temperature. \( \text{NaOH} \) (1 M, 1.0 mL) was added to each sample followed by the addition of deionized water (1.0 mL). Absorbance of the orange color developed was recorded at 510 nm using UV/Vis spectrometer (Hitachi-Japan U 2910). The extract was prepared in the same manner. Using the standard curve, milligrams of quercetin equivalents per dry weight of the extract (QE/g) was obtained.

### 3. Results and Discussion

#### 3.1. In Vitro \( \alpha \)-Amylase Inhibitory Activity

Phytochemicals have been proven to possess the ability to inhibit starch-hydrolyzing enzymes and thereby restrain glucose release from starch and its subsequent absorption. Due to this reason, the interest to search for new therapeutic drugs of plant origin has increased over recent times [8]. Kinetic studies on plant extracts have revealed that the inhibition of \( \alpha \)-amylase by phytochemicals occur via uncompetitive, noncompetitive, or competitive mechanisms [4, 8, 29]. During the \( \alpha \)-amylase inhibitory assay, FLAE exhibited an increased inhibition on the enzyme activity with the increasing concentrations upon the introduction to the starch-enzyme mixture as shown in Figure 1. Hence, the conversion of starch to glucose is inhibited in the reaction media, and the enzyme activity is blocked by FLAE in a concentration-dependent manner. At 400 \( \mu \)g/mL concentration, FLAE exhibited its highest inhibitory activity of 90%, and the percentage inhibition varied from 29%–90% throughout the series of concentrations (2.5–400 \( \mu \)g/mL) of FLAE. The overall results obtained for the *in vitro* \( \alpha \)-amylase inhibitory assay indicates that FLAE possesses a considerable enzyme
Inhibitory activity, and the potency is as good as the standard drug acarbose. Acarbose is the standard noninsulinotropic glycoside inhibitor drug in the market, and it competitively inhibits the conversion of oligosaccharides to monosaccharides by \( \alpha \)-glucosidases present in the brush border membrane of the small intestine due to structural similarity with natural oligosaccharides [30]. According to the literature, it is reported that acarbose has adverse effects in the gastrointestinal tract, such as diarrhea and flatulence, due to extreme inhibition of the enzymes upon consumption [31]. Also, it is reported that the use of acarbose together with dietary control may lead to hepatitis and also extreme inhibition of pancreatic glycosidases will eventually lead to digestive disorders followed by abnormal bacterial fermentation in the colon [32]. Plant-based remedies are sort to combat diseases due to its lack or minimal toxic effects. Therefore, FLAE could be identified as a potential \( \alpha \)-amylase inhibitor with considerably good inhibitory activity.

3.2. In Vitro Protein Glycation Inhibitory Activity. Glycation is a regular process that occurs in our body at a very low rate and is also a major cause of aging [11]. The process mainly involves a nonenzymatic reaction between reducing sugars and protein residues, which were first identified by Maillard in the early 1900s [12]. The importance of this reaction in identifying diabetes was realized many years after that. It was found that the levels of hemoglobin A1c (HbA1c), a naturally occurring minor species of hemoglobin in humans, were elevated in the patients with diabetes, and hence, this is considered as a measurement of identification of diabetes mellitus [11]. Elevated blood glucose levels will increase the protein glycation process, which is the reaction of formation of Amadori product, which will eventually lead to the formation of advanced glycation end products. Compared to glucose, fructose can form glycated products at an increased rate [1]. Role of fructose was identified as to glycate the proteins in a non-enzymatic manner or to activate the polyol pathway, which will enhance the formation of fructose from glucose. Additionally, AGEs resulted from fructose leads to significantly higher fluorescence intensities compared to those resulted from glucose, according to previously published articles [33].

In the presence of elevated sugar levels, the formation of the glycation end products accelerates and hence becomes responsible for the secondary complications in patients with diabetes mellitus [1]. Prolonged hyperglycemia may eventually lead to chronic diabetic complications, such as diabetic neuropathy, nephropathy, retinopathy, and cardiovascular diseases [1, 5, 8]. Aminoguanidine, which was utilized as the positive AGE inhibitor in this study, has been shown to inhibit the AGE formation by trapping reactive dicarbonyl species formed prior to the Amadori products forming AGEs [13, 34]. Due to the toxic effect of the synthetic aminoguanidine, which was the first anti-AGE drug, plant-based remedies were sought for which had fewer side effects.

Formation of AGEs was observed after a week of incubation of BSA and fructose in buffer solutions at room temperature by measuring the augmentation of fluorescent intensity in BSA glycated with fructose. The fluorescent intensity increased considerably throughout the period, and the introduction of FLAE (15.6–250 \( \mu \)g/mL) to the reaction mixtures demonstrated a drastic reduction in the fluorescent intensity of the mixtures (Figure 2(a)). It is significant that the FLAE showed 98% inhibitory potential toward AGE formation at a concentration of 250 \( \mu \)g/mL added to the solutions as indicated in Figures 2(a) and 2(b). Similar to the effect of FLAE, aminoguanidine (250 \( \mu \)g/mL and 750 \( \mu \)g/mL) also exhibited a significant reduction in fluorescent AGEs formation when introduced to BSA-fructose medium. Percentage inhibition of AGE at 250 \( \mu \)g/mL and 750 \( \mu \)g/mL was 95% and 99%, respectively.

3.3. In Vitro Antioxidant Activity. Previous studies have unveiled the relationship between antioxidants and non-enzymatic protein glycation [35, 36]. Imbalance in ROS and antioxidants in the body could lead to many diseases including diabetes mellitus. Diabetes mellitus is closely associated with oxidative stress, and it has been suggested that oxidation reactions could accelerate the formation of AGEs [35]. Formation of AGE occurs through reactive oxygen species (ROS) apart from the radical yielding reactions and other reactive intermediates that could occur due to Maillard reaction [36]. Therefore, treatment with antioxidant agents is one of the therapeutic insights that have been proposed. In vitro antioxidant activity of FLAE was obtained using DPPH radical scavenging assay. FLAE (25–200 \( \mu \)g/mL) demonstrated a significant free radical scavenging activity, which increased with the increasing concentrations (26%–60%). Similar to the effect of FLAE (25–200 \( \mu \)g/mL), a considerable scavenging activity was observed in reaction mixtures with BHT (15%–76%). As indicated in Figure 3, percentage scavenging activities obtained.
for both FLAE and BHT depict that FLAE show higher activity toward free radical scavenging compared to the respective concentration of BHT.

The radical scavenging activity of plants is associated with polyphenolic compounds and more closely with flavonoids [2, 14]. FLAE unveiled a total phenolic content of $198 \pm 7.2 \, \text{mg GAE/g}$ toward the Folin–Ciocaltue assay and a total flavonoid content of $81.2 \pm 1 \, \text{mg QE/g}$ for aluminium chloride assay. According to the results obtained, FLAE exhibited high TPC and TFC values. Phenolics and flavonoids are also responsible for antioxidative properties, and the development of food supplements and pharmaceutical products with antioxidants has become one of the major concerns recently [36]. Therefore, according to the literature, extracts with both antioxidant and antiglycations properties have shown high efficacy toward the treatment of diabetes mellitus [37]. The idea is that reactive carbonyl species formed during AGE formation could be scavenged by natural agents rich with antioxidants [36]. Therefore, the observed high antiglycation activity and α-amylase activity could be due to the presence of specific compounds or due to the synergistic effect of antioxidants, phenolic compounds, and flavonoids [38, 39]. Additionally, the previous study by Ellepola et al. [22] that FLAE is nontoxic adds value to the results we presented in this article, suggesting therapeutic benefits that can be achieved for diabetes-related complications by administering FLAE with minimal toxicity.

4. Conclusion

The present study scientifically validates the use of FLAE (which was prepared according to the method of preparation of “kasaya” in traditional medicine) as a remedy for diabetes.
We have proven that FLAE not only inhibits α-amylase but also inhibits advanced glycation end product formation, which in turn would prevent complications related to diabetes. This extract also possesses significant antioxidant activity, and hence, it has good potential as a remedy for many diseases, which are thought to arise due to oxidative stress.

**Data Availability**

The data used to support the findings of this study are available from the corresponding author upon request.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**References**


