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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

elevated blood sugar levels. Accumulations of glycation end products are concomitant with various diseases [11]. Chronic hyperglycemia resulting in AGEs facilitates diabetes-related complications, such as diabetic retinopathy, nephropathy, cataract, and atherosclerosis [2, 6, 10–12]. Aminoguanidine is the first inhibitor of AGEs discovered in 1986 [12, 13]. However, it may have serious toxicity when administered for diabetic nephropathy [1, 2].

The nonenzymatic reaction of sugars and free amino groups in amino acids or proteins form Schiff base structures. This process is called the Maillard reaction or advanced glycation [12, 13]. These Schiff bases are unstable; hence, they rearrange to form Amadori products via chemical transformations. These Amadori products undergo further rearrangements followed by other reactions such as cyclization, oxidation, and dehydration and cross-linking to form Advanced Glycation End products (AGEs) [12–15].

There is considerable interest in search of plant-based remedies with antiglycation activity, as they may potentially inhibit AGE formation resulting in delaying and preventing the onset of diabetic complications with minimal side effects. As in the case of many diseases, free radicals have also been implicated in the pathway of AGE formation [2, 13, 14].

Flueggea leucopyrus Willd (FLAE) (Katupila in Sinhala, Irubulai, Mudbulanji in Tamil) is a medicinal plant used for the treatment of various types of diseases in the Ayurvedic system of medicine in Sri Lanka as well as in other Asian countries [16, 17]. *Flueggea leucopyrus* Willd is a large bush with long, straggling, thorny branches and small, globose, white berries [16]. *Flueggea leucopyrus* Willd has proven to be very effective in the treatment of different diseases in Ayurveda medicine, such as cough, hay asthma, bowel complaints, laxatives, diarrhea, gonorrhea, constipation, mental illness, kidney stones, and cancer [18–20]. The plant has reported high disinfectant activity [17], and hence, the hydroalcoholic extract has been assessed for its antimicrobial potential against *Serratia marcescens*, *Salmonella typhi*, *Escherichia coli*, and *Staphylococcus mutant* [19, 21]. The diuretic activity of the aqueous extract of *Flueggea leucopyrus* Willd has also been studied using rat models, and during this study, it has been revealed that there is no adverse liver or renal toxicity in rats due to the extract [22].

The objective of this research is to scientifically validate the folklore belief that *Flueggea leucopyrus* Willd could be used as a remedy for diabetes. This article addresses not only on the fact that FLAE can inhibit α-amylase enzyme but also on the fact that it controls AGE formation which could delay the onset of diabetes complications.

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 hydroxyl toluene 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 (I%) of α-amylase was determined as follows:

$$I\% = \frac{(A_C - A_{CB}) - (A_T - A_{TB})}{(A_C - A_{CB})} \times 100\%, \quad (1)$$

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 μg/mL) a commercially available α-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 μg/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:

$$I\% = \frac{(F_C - F_{CB}) - (F_S - F_{SB})}{(F_C - F_{CB})} \times 100\%, \quad (2)$$

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$^{\cdot}$ by the antioxidants present in the aqueous extract of *Flueggea leucopyrus* Willd was carried out according to a method discussed by Kuganesan et al. [28]. The test samples (25–200 μg/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:

$$I\% = 1 - \left(\frac{A_S}{A_C}\right) \times 100\%, \quad (3)$$

where A_S is the absorbance of the sample and A_C is the absorbance of the control. Butylated hydroxy toluene (BHT) (25–200 μg/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 leucopyrus* Willd was determined by Folin–Ciocalteu (FC) method according to Kamtekar et al. [25]. Different concentrations of gallic acid (20–90 μg/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. Na_2CO_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 $NaNO_2$ (5% w/v, 0.15 mL). Reaction mixtures were incubated at room temperature for 5 minutes. Then $AlCl_3$ solution (10% w/v, 0.15 mL) was added to each mixture, mixed well, and allowed to stand for 6 minutes at room temperature. 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 α-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 α-amylase by phytochemicals occur via uncompetitive, noncompetitive, or competitive mechanisms [4, 8, 29]. During the α-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 μg/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 μg/mL) of FLAE. The overall results obtained for the *in vitro* α-amylase inhibitory assay indicates that FLAE possesses a considerable enzyme

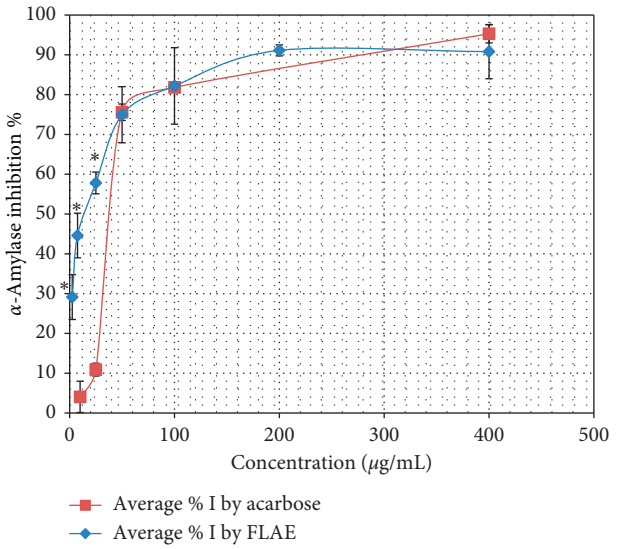


Figure 1: Overlay of the dose-response curves for percentage inhibition of α-amylase activity of FLAE in comparison with the positive control acarbose. According to the results obtained, FLAE and acarbose show similar activity above 50 μg/mL concentration; but at lower concentrations, the activity of FLAE is significantly high. The results are presented as mean ± standard deviation of three independent experiments.

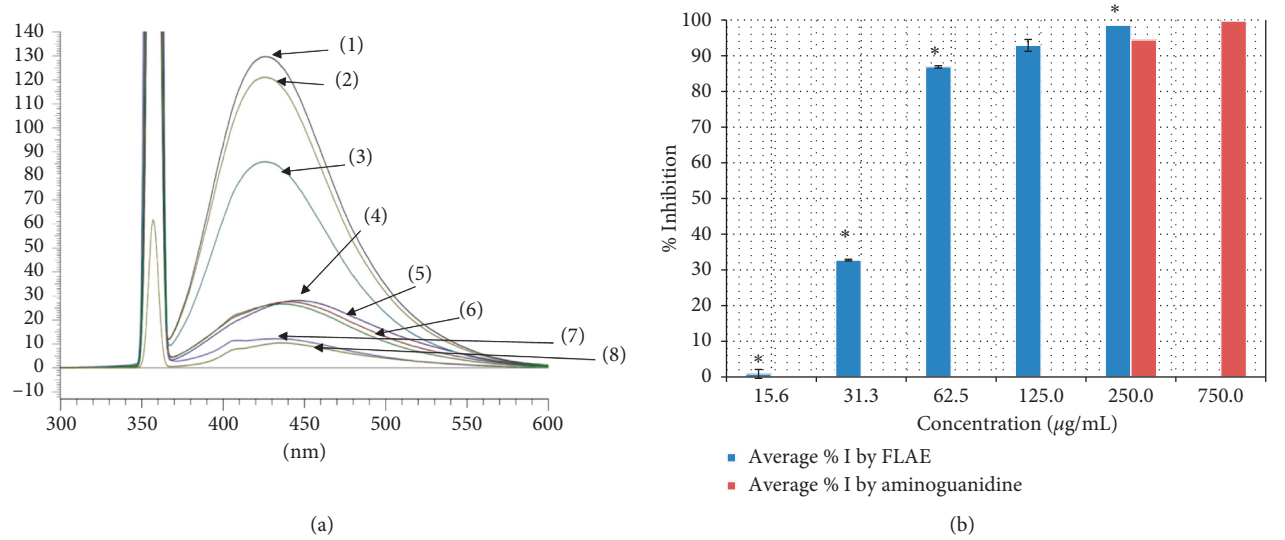
inhibitory activity, and the potency is as good as the standard drug acarbose. Acarbose is the standard noninsulinotropic glycoside inhibitor drug in the market, and it competitively inhibits the conversion of oligosaccharides to monosaccharides by α-glucosidases present in the brush border membrane 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 α-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 nonenzymatic 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 amnioguanindine, 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 μg/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 μg/mL added to the solutions as indicated in Figures 2(a) and 2(b). Similar to the effect of FLAE, aminoguanidine (250 μg/mL and 750 μg/mL) also exhibited a significant reduction in fluorescent AGEs formation when introduced to BSA-fructose medium. Percentage inhibition of AGE at 250 μg/mL and 750 μg/mL was 95% and 99%, respectively.

3.3. In Vitro Antioxidant Activity. Previous studies have unveiled the relationship between antioxidants and nonenzymatic 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 Mailard 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 μg/mL) demonstrated a significant free radical scavenging activity, which increased with the increasing concentrations (26%–60%). Similar to the effect of FLAE (25–200 μg/mL), a considerable scavenging activity was observed in reaction mixtures with BHT (15%–76%). As indicated in Figure 3, percentage scavenging activities obtained



(a) (b)

Figure 2: (a) The overlaid fluorescence emission spectra of the samples after a week that contained (1) BSA + Frc (fructose), (2) BSA + Frc + FLAE (15.6 μg/mL), (3) BSA + Frc + FLAE (31.25 μg/mL, (4) BSA + Frc + FLAE (250 μg/mL), (5) BSA + Frc + FLAE (125 μg/mL), (6) BSA + Frc + FLAE (62.5 μg/mL), (7) BSA only, (8) Frc only. (b) Comparison of percentage inhibition of fluorescent AGEs by FLAE and aminoguanidine. For concentrations at 62.5 μg/mL and above, FLAE showed an average percentage inhibition higher than 50%. At concentrations of 62.5 μg/mL and below, FLAE showed a significantly lower activity than aminoguanidine at 250 μg/mL. However, at 250 μg/mL concentration, FLAE showed a significantly higher activity compared to aminoguanidine at the same concentration. $^*p < 0.05$ between %I of FLAE and I% of AG at 250 μg/mL. The results are presented as mean ± standard deviation of three independent experiments. In certain cases, error bars cannot be visualized due to the values being extensively small.

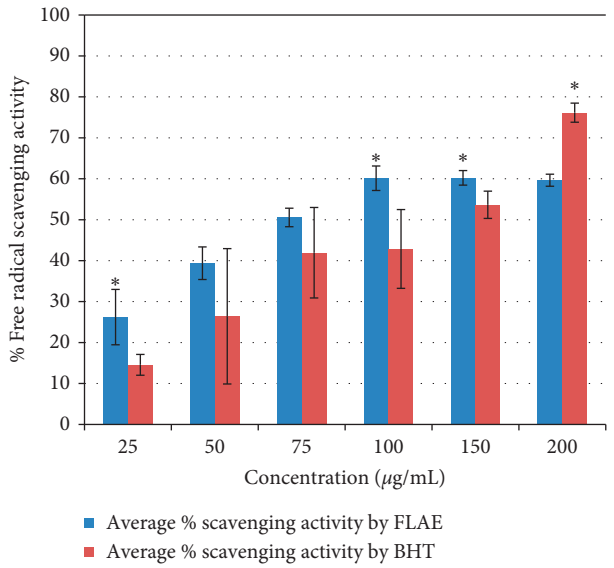


Figure 3: Comparison of percentage free radical scavenging activity of FLAE and BHT. FLAE showed significantly high free radical scavenging activity compared to BHT at 25, 100, and 150 μg/mL concentrations, whereas at 200 μg/mL concentration, the activity of BHT was significantly high for the DPPH assay. $^*p < 0.05$ between FLAE and BHT at similar concentrations. The results are presented as mean ± standard deviation of three independent experiments.

for both FLAE and BHT depicts 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 ± 7.2 mg GAE/g toward the Folin–Ciocaltue assay and a total flavonoid content of 81.2 ± 1 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.

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