Fractionation of *Dioscorea bulbifera* bulbil protein, its antioxidative potential and inhibitory effects on Carbohydrate-hydrolyzing enzymes

Olayemi Oludele ODEKANYIN 1*, PhD; Fehintoluwa Deborah AKINYEMI 1; MSc student; Omodolapo Eniola BADMUS 1; MSc student

1 Biochemistry and Molecular Biology Department, Faculty of Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria.

Abstract

The study fractionated *Dioscorea bulbifera* bulbil proteins, determined the fractions inhibitory potential against α-amylase and α-glucosidase activity, and evaluated their antioxidative activity in-vitro. *Dioscorea bulbifera* bulbil flesh was homogenized in Phosphate Buffer Saline (pH 7.2), centrifuged at 10,000 xg for 30 min and supernatant was subjected to ammonium sulphate fractionation. Three fractions (25%, 60% and 90%) were produced and their hemagglutinating activity, blood group and sugar specificity were determined. Ability of the fractions to inhibit α-amylase and α-glucosidase activity was investigated. Antioxidative properties were evaluated using DPPH, Nitric oxide and hydroxyl radicals scavenging assay. Their reducing power, total antioxidative capacity and ferrous chelating activity were also evaluated. Data obtained revealed the presence of lectin in the crude protein extract, 25% and 60% protein fractions. The lectin agglutinated rabbit blood erythrocyte better than human blood groups erythrocyte. Protein fraction (60%) has no hemagglutinating activity towards human blood erythrocyte. Mannose completely inhibited the hemagglutinating activity. Alpha-amylase and α-glucosidase activities were inhibited by 25% and 60% protein fractions with no significant difference. But their inhibitory action was better than that of acarbose. All the free radicals tested were scavenged by the fractions to various degrees. Sixty percent protein fraction could not scavenge nitric oxide but it was a better scavenger of hydroxyl radical. No activity was recorded for 90% protein fraction. The results showed the anti-diabetic potential of the protein fractions and can be harnessed for possible development of therapeutics agents for the treatment of diabetes and oxidative stress associated ailments.

Keywords: Diabetes, antioxidants, α-amylase, α-glucosidase, Fractions, *Dioscorea bulbifera*, bulbil

1. Introduction

*Diabetes mellitus* is the most serious, chronic metabolic syndrome, characterized by high blood glucose levels (1) and caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced (2). The alarming rate at which this disorder is increasing has made World Health Organization (WHO) to project that by 2030, diabetes would become seventh leading cause of death globally (3). One therapeutic approach to lower blood glucose levels is to retard the absorption of glucose via inhibition of enzymes, such as α-glucosidase and α-amylase, in the digestive organs (4, 5). Also, insulin therapy has been shown to be another satisfactory approach in the treatment and management of *Diabetic mellitus*. Antioxidants also have the potentials to ameliorate the disturbance caused by free radicals produced during oxidative stress in diabetes. Increase in free radicals’ amount is closely associated with the glucose oxidation, lipid peroxidation and non-

Corresponding Author: Olayemi Oludele ODEKANYIN, Biochemistry and Molecular Biology Department, Faculty of Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. Email: dekanyin@oauife.edu.ng

enzymatic glycation of protein which influence the diabetes mellitus (6). Natural antioxidants are abundant in plants and due to the various complications in the existing drugs for the treatment of diabetic, natural antioxidants are being employed for diabetic management. World Health Organization (WHO) also advocates the use of herbal medicines over the drugs currently in use for treatment of diabetes (7). Recently, focus has been on proteins that have the ability to reduce the postprandial hyperglycemia though the mechanism is not yet known. Wahab et al., (8) concluded that proteins derived from Pleurotus pulmonarius basidiocarps have a significant potential as alternatives in treating type 2 diabetic mellitus based on their ability to inhibit α-glucosidase and α-amylase activities in vitro. Also, protein from the seed coat of Canavalia ensiformis showed hypoglycemic activity (9). A class of protein known to agglutinate red blood cell, lectins, have also been shown to exert hypoglycemic effects in-vivo (10, 11). In 2017, a protein was isolated from the leaf of Moringa oleifera and was shown to possess hypoglycemic and antioxidant potential in Alloxan-Induced Diabetic Mice (12).

Dioscorea bulbifera, the air potato, is a species of true yam native to Africa, China, Japan, Northern Australia, Southern Asia, Indonesia and India with slight differences between those found in each place. It is a large creeper, 6 meters (20ft) or more in length. It is a monocotyledonous dioecious herbaceous perennial climber with broad leaves and two types of storage organ. It has been reported that D. bulbifera is being used in different countries of the world as traditional medicine to treat several diseases such as parasitic infections, microbial infections, oxidative stress and degenerative diseases, diabetes and digestive problems (13). Similarly, its extracts are reported to possess antihyperlipidemic, antitumor, anorexiant, analgesic, anti-inflammatory, plasmid curing, and anti-hyperglycemic effects (14). Though the antidiabetic potential of D. bulbifera has been reported (15, 16), the activity was linked to a bioactive component named diosgenin. Diosgenin is secondary metabolite obtainable only through organic solvent extraction and reported to possess various pharmacological activities (17). Literature search shows that there are no reports on the D. bulbifera bulbil protein antidiabetic activity. This study intends to investigate the in vitro therapeutic potential of bioactive proteins in D. bulbifera bulbils in controlling the blood glucose levels through inhibition of carbohydrate-hydrolyzing enzyme and free radical generation.

2. Material and Methods

2.1. Sample collection

Dioscorea bulbifera bulbils were collected in the month of August from Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The plant and the bulbils were identified at the Ife Herbarium, Botany Department, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

2.2. Preparation of Crude Extract

Dioscorea bulbifera bulbils (aerial tuber) were peeled. Then, 25.84g of peeled bulbils was homogenized in 100ml of phosphate buffer saline (PBS, pH 7.2) and stirred for 4 hours on an electric magnetic stirrer at 4°C. The mixture was centrifuged at 10,000 xg for 30 min. The supernatant which constitutes the crude protein extract was subjected to ammonium sulphate fractionation. The first protein fraction was obtained by subjecting the crude protein extract solution to 25% ammonium sulphate saturation. The precipitate was collected 24 hrs later and represents the third protein fraction (90% fraction) and the supernatant of the second precipitation was also brought to 60% ammonium sulphate saturation. The precipitate was also collected and represents the second protein fraction (60% fraction) and the supernatant of the second precipitation was also brought to 90% saturation. The precipitate was collected by centrifugation and represents the third protein fraction (90% fraction). All the precipitates were dialyzed extensively and the dialysates were freeze-dried separately. The freeze-dried dialysates were used for various assays.

2.3. Protein Concentration Determination

Protein concentration of the protein fractions were determined using the method described by Lowry et al. (18) and Bovine Serum Albumin (BSA) served as standard protein.

2.4. Hemagglutinating Activity

2.4.1. Glutaraldehyde fixation of red blood cells

In order to fix the erythrocytes, the blood...
samples were collected from human and animal into heparinized bottles. It was immediately centrifuged at 3,000 rpm using a table-top centrifuge (Hospibrand 0502-1) for 15 min to obtain the erythrocytes. The erythrocytes were washed five times with PBS and then fixed in chilled 1% glutaraldehyde-PBS solution for 1 hour at 4 °C with intermittent mixing. After the fixation, the mixture was centrifuged for 5 min at 3,000 rpm to collect fixed erythrocytes. The fixed erythrocytes were extensively washed with PBS to remove the glutaraldehyde. Two percent of the erythrocytes were prepared in PBS containing 0.02 % sodium azide. This was stored in a refrigerator for further use.

2.4.2. Hemagglutinating assay

The presence of lectin in the crude extract of *D. bulbifera* bulbil and various fractions was determined through modified hemagglutinating assay procedure of Wang et al. (19). The assay was done in a 96-well U-shaped microtitre plate. Phosphate Buffered Saline (100 μl) was pipetted into all the wells of the microtiter plate sequentially. After this, 100 μl of the crude extract or fractions was added to the first well in the first row and the mixture was serially diluted up to the 24th well (2 rows). Fifty microliter of the fixed erythrocyte suspension (50 μl) was added to each well. The plate was left undisturbed for two hours on the laboratory bench. It was later observed for any visible hemagglutination. The control experiment was without extract or any of the fractions. Hemagglutination titre unit was taken as the highest dilution reciprocal of the crude extract or any of the fractions producing visible hemagglutination.

2.4.3. Sugar specificity test

Sugar specificities of *D. bulbifera* bulbil crude protein and protein fractions were determined by comparing the abilities of different sugars to inhibit the hemagglutinating activity of the protein fractions. Firstly, the serial dilution of the protein fractions was carried out as described in section 2.4.2 until the last dilution where hemagglutination was observed. Sugar solution (0.2 M, 50 μl) was added into each well, while PBS substituted sugar solution in control wells. The microtitre plate was then incubated at room temperature for 2 hrs. Erythrocyte suspension (50 μl) was added into each well. The plate was left undisturbed for two hours on the laboratory bench and then observed for any visible hemagglutination. The tested sugars were: glucosamine HCl, lactose, maltose, sorbose, sucrose, mannitol, galactose, N-acetyl-D-glucosamine, arabinose, glucose, mannose and 2-deoxy-D-glucose.

2.5. Enzymes Inhibition Assays

2.5.1. Alpha-amylase inhibitory activity (In-vitro)

Ability of the protein fractions (25%, 60% and 90%) to inhibit the α-amylase activity was investigated following a modified method of Ademiluyi et al., (20). The reacting component includes same volume (500 μL) of phosphate buffer (0.02 M, pH 6.9), 1.5% α-amylase (prepared in the Phosphate buffer) and varying concentrations of various protein fractions (0.005 – 0.25 mg/ml). The mixture was placed in a water bath set at 25°C for half an hour. Addition of 500 μL 1% soluble potato starch solution as substrate to the reaction mixture preceded another incubation at the same temperature but for 10 min. After this, the reaction was terminated by addition of 500 μL 3, 5-dinitrosalicylic acid (DNS) color reagent. The entire experiment was then boiled for 5 min and cooled under running tap water. Distilled water (2.5 mL) was pipetted into each tube before measuring the absorbance at 540 nm. Acarbose at the same concentration range was employed as a positive control. Control that represents 100% amylase activity was set up in a parallel experiment and it contains a buffer instead of protein fractions. The level of inhibition by the fractions was calculated in percentage as follows in equation 1.

\[
\%\text{Inhibition} = \frac{Abs(\text{control}) - Abs(\text{sample})}{Abs(\text{control})} \times 100%
\]

(Eq. 1)

Abs<sub>sample</sub> is the absorbance in the presence of test substance and Abs<sub>control</sub> is the absorbance of control.

2.5.2 In vitro alpha-glucosidase inhibitory activity

Shai et al. (21) earlier described the method for measuring α-glucosidase activity inhibition by various agents. The method was adopted in determining the potential of the protein fractions of *D. bulbifera* bulbil to inhibit the activity of α-glucosidase *in vitro* but it was modified. The experiment was carried out in a microtiter plate with 96 wells. Twenty microlitres of each
of the following: phosphate buffer (100 mM, pH 6.8), α-glucosidase prepared in the phosphate buffer, varying concentration of the protein fractions (0.005-0.05 mg/ml) and the substrate (2.5 mM p-nitrophenylglucopyranoside) were mixed in the microtiter plate. The plate was placed in a water bath for incubation at 37 °C for 15 min. Addition of 80 µL 0.2 M Na₂CO₃ to the reaction mixture terminates the reaction. The microtiter plate was placed in the microtiter plate reader for absorbance reading at 405 nm. The absorbance showed the amount of p-nitrophenol released. Control experiment that shows 100% α-glucosidase activity was performed in the absence of protein fractions. In addition, positive control where a standard drug acarbose was used at the same concentration range was set up along with the experiment. The inhibition of α-glucosidase activity by the protein fractions was expressed in percentage using the equation 2.

\[
\%\text{Inhibition} = \frac{\text{Abs} \text{(control)} - \text{Abs} \text{(sample)}}{\text{Abs} \text{(control)}} \times 100\%
\]  
(Eq. 2)

Where, \(\text{Abs}_{\text{sample}}\) is the absorbance in the presence of test substance and \(\text{Abs}_{\text{control}}\) is the absorbance of control.

2.6. In-Vitro Antioxidant Activities

2.6.1. Assay of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging

Brand-Williams et al. (22) procedure of DPPH radical scavenging assay was used to determine the ability of the protein fractions to change the deep violent colour of the DPPH solution to yellow. The colour change measured at 517 nm spectrophotometrically indicates the level of DPPH radical scavenging ability of the fractions. Equal volume (0.15 ml) of the protein fractions/standard (ascorbic acid) and 0.3 mM DPPH in methanol were mixed in a 96-well micro-titre plate. The plate was placed in a dark room for half an hour after which the absorbance was taken at 517 nm. DPPH solution and methanol without protein fractions/standard was used as control. The inhibition of colour change in percentage was calculated with the formula below in equation 3.

\[
\text{DPPH radical scavenging(%) =} \frac{\text{Abs} \text{(blank)} - \text{Abs} \text{(sample)}}{\text{Abs} \text{(blank)}} \times 100\%
\]  
(Eq. 3)

2.6.2. Assay of hydroxyl (OH) radical scavenging

Potential of the protein fractions to scavenge hydroxyl radical produced from the Fe³⁺/ascorbate/EDTA/H₂O₂ system ahead of deoxyribose was determined according to the method of Halliwell et al. (23). Working reagent was prepared in 10 mM phosphate buffer (pH 7.4). The constituent includes 0.1 mM ascorbic acid, 3 M deoxyribose, 0.1 mM EDTA, 2 mM H₂O₂ and 1 mM FeCl₃·6H₂O. One milliliter of various concentrations of the protein fractions (0 - 350 µg/ml) was mixed with 1 ml of working reagent, incubated for 60 min at 37 °C. Thereafter, 1 ml each of 1% (w/v) thiobarbituric acid (TBA, prepared in 0.25N HCl) and 10% (w/v) trichloro-acetic acid was added to the mixture in the tubes. Water bath was set at 100 °C and the tubes was dip in it for 20 mins. The absorbance of the chromogen developed and extracted into 1 ml of butan-2-ol was measured at 532 nm against blank. The inhibition percentage was evaluated with the equation 4.

\[
\%\text{(OH)Inhibition} = \frac{\text{Abs} \text{of control} - \text{Abs} \text{of sample}}{\text{Abs} \text{of control}} \times 100\%
\]  
(Eq. 4)

2.6.3. Nitric oxide (NO) radical scavenging assay

Amended assay protocol of Green et al. (24) was used to determine the protein fraction’s ability to inhibit nitric oxide radical generation. Sodium nitroprusside (2.5 mM) prepared in phosphate buffer saline and different concentration (0.0 – 5.0 mg/ml) of the protein fractions were mixed in test tubes in ratio 9:1, respectively. The mixture was incubated under illumination for two and half hours. Thereafter, 0.5 ml each of 1% sulphanilamide prepared in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride (NEI) was added to the mixture one after the other with 10 mins incubation in the dark room interval between them. Absorbance measurement of the deepness of the chromophore formed was done at wavelength of 546 nm. Nitric oxide radical generation inhibition percentage was evaluated with the formula 5.

\[
\%\text{NOInhibition} = \frac{\text{Abs} \text{of control} - \text{Abs} \text{of sample}}{\text{Abs} \text{of control}} \times 100\%
\]  
(Eq. 5)
2.6.4. Total Antioxidant Capacity (TAC) assay

Reduction of molybdate (VI) to molybdate (V) by the protein fractions was measured using Prieto et al. (25) procedure. Reagent solution (containing 4 mM ammonium molybdate, 28 mM sodium phosphate and 0.6 M sulphuric acid) was mixed with protein fractions/standard solution (ascorbic acid) in ratio 10:1 in test tubes. The mixture in the test tubes was later incubated at 95°C for one and half hour in a regulated water-bath. The absorbance was measured at wavelength 695 nm after the reacting mixture has cooled to room temperature. Distilled water replaced the protein fractions in the reacting mixture that served as control. The antioxidant activities of the protein fractions were expressed as an ascorbic acid equivalent per gram of the protein fractions.

2.6.5. Ferric Reducing Antioxidant Power (FRAP) assay

To measure the reducing antioxidant power of the protein fractions, Benzie and Strain (26) procedure was employed with little amendment. Three hundred millimolar of acetate buffer (pH 3.6) was prepared. Also, 10 mM 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) and 20 mM FeCl3.6H2O were separately prepared. The solutions were mixed at point of use in ratio 10:1:1 respectively, to obtain the FRAP working reagent. The reacting mixture for the assay contain 50 µl of protein fractions/standard solution (ascorbic acid) in concentration range of 0-100 µg/ml and 1 ml of FRAP working reagent. The reaction was allowed to proceed for 10 min, thereafter absorbance reading was measured at 593 nm against reagent blank. The reacting mixture was shaded from direct sunlight when taken the reading. The ferric reducing antioxidant power of the protein fractions was expressed as ascorbic acid equivalent.

2.6.6. Ferrous-ion chelating activity assay

Evaluation of the ability of the protein fractions to chelate ferrous ion was carried out according to the amended assay procedure of Singh and Rajini (27). Reagents used are 2 mM FeCl3.4H2O and 5 mM Ferrozine which was diluted 20 folds. The reaction started with mixing of 1 ml of different concentration of protein fractions with 1 ml FeCl3.4H2O. The reaction proceeded for 5 mins at ambient temperature followed by the addition 1 ml ferrozine solution.

The reacting mixture was vortexed and incubated for 10 mins. Absorbance of the mixture was taken at 562 nm. Chelating effect percentage was expressed as equation 6,

\[
\% \text{Inhibition} = \frac{(Abs_{\text{control}} - Abs_{\text{sample}})}{Abs_{\text{control}}} \times 100\%
\]

(Eq. 6)

2.7. Data analysis

Results were subjected to statistical analysis performed with GraphPad Prism Version 6.0 (Graphpad Software Inc., San Diego, California). The experimental values were shown as Mean ± Standard Error of Mean where n=3. One-way ANOVA was used to examine multiple comparisons. The level of significance was \( p < 0.05 \).

3. Result

3.1. Protein concentration

The soluble protein of D. bulbifera bulbil was extracted using PBS (pH 7.2) and further fractionated with ammonium sulphate. The amount of soluble protein present in crude extract is 12.53 mg protein per milliliter of crude extract solution. The amount of soluble protein fractionation yielded 6.1 mg protein for 25% fraction, 4.21 mg protein for 60% fraction and 0.98 mg protein for 90% fraction. Protein in 25% fraction is about 50% of total crude protein extract. Sixty percent fraction con-

<table>
<thead>
<tr>
<th>Source of Erythrocyte</th>
<th>Hemagglutinating Activity</th>
<th>Crude extract</th>
<th>25% Fraction</th>
<th>60% Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human A</td>
<td></td>
<td>2(^3)</td>
<td>2(^2)</td>
<td>2(^0)</td>
</tr>
<tr>
<td>Human B</td>
<td></td>
<td>2(^2)</td>
<td>2(^3)</td>
<td>2(^0)</td>
</tr>
<tr>
<td>Human O</td>
<td></td>
<td>2(^3)</td>
<td>2(^3)</td>
<td>2(^0)</td>
</tr>
<tr>
<td>Human AB</td>
<td></td>
<td>2(^2)</td>
<td>2(^2)</td>
<td>2(^0)</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td>2(^7)</td>
<td>2(^6)</td>
<td>2(^3)</td>
</tr>
</tbody>
</table>
tained about 34% while 90% have 8%.

3.2. Hemagglutinating Activity

Presence of lectin was detected in the crude protein extract and also in the protein fractions. Crude protein extract and 25% fraction had better preference for rabbit erythrocytes agglutination when compare to ABO blood grouping of human. Sixty percent (60%) protein fraction only agglutinated rabbit erythrocyte with low hemagglutination titre (23) (Table 1). It was established that the lectin present in the 25% fraction is mannose specific (Table 2).

3.3. Carbohydrate –hydrolyzing enzyme activity inhibition

The results obtained revealed the efficacy of the protein fractions in inhibiting the activity of two carbohydrates-hydrolyzing enzymes assayed. Protein fractions (25% and 60%) inhibited the enzymes better than acarbose, a standard inhibitor. The results also showed that α-glucosidase was better inhibited by the fractions (Table 3). Inhibition of α-amylase by 25% and 60% protein fractions were not significantly different. The same occurrence was noticed for α-glucosidase. Figure 1, Figure 2, and Table 3 showed the enzymes inhibition results and their Half-maximal inhibitory concentration (IC₅₀). The α-amylase and α-glucosidase inhibition was directly proportional to the concentration of the protein fractions (Figure 1 and 2). The third protein fraction (90%) exhibited/showed no enzyme inhibitory activity.

3.4. Antioxidant activity

Table 4-7 and Figure 3 depicted the antioxidant activity assay results of the various methods used. Table 4 revealed that the protein fractions,
25% and 60%, were able to scavenged DPPH radical in a dose dependent way, though their ability is significantly different from the standard radical scavenging agent (ascorbic acid). The IC$_{50}$ for the ascorbic acid and fractions are shown in Table 4. Aside the ability to scavenge DPPH, the protein fractions also scavenged hydroxyl radical but 60% fraction was a better scavenger of OH radical. Its ability was also higher than one display by ascorbic acid (Figure 3).

Nitric oxide (NO) radical formation was inhibited by 25 % protein fraction with IC$_{50}$ of 1.3 ± 0.06 mg/ml; result that is significantly different from ascorbic acid used as standard (Table 5). 60% fraction concentration below 2.0 mg/ml could not inhibit the NO formation while 90% fraction showed no inhibition even at high concentration of 10 mg/ml.

Table 6 showed that protein fractions possessed considerable potential to reduce molybdenum (IV) to molybdenum (V) leading to the molybdenum (V) complex formation with 25% fraction having higher value. They also exhibited ability to reduce Fe$^{3+}$ to Fe$^{2+}$ as shown in Table 7. It was deduced that the protein fractions were found to inhibit ferrozine-Fe$^{2+}$ complex formation. The ability increases as the concentration of fractions goes up. 25% exhibited a better inhibitory action, though below the value of ascorbic acid.

### 4. Discussion

The protein fractions of the *D. bulbifera* bulbils were obtained by ammonium sulphate fractionation. This is usually done by modifying the solvent conditions and taking advantage of the variations in solubility of the target protein relative to other proteins and macromolecules present in a crude extract. Researchers commonly employ differential precipitation of protein with ammonium sulphate at the initial stages of protein purification either from animals or plants sources (28). It is also used to concentrate protein towards the last stages of purification. The amount of protein obtained from the crude extract of *D. bulbifera* bulbils was found to be in accordance with the report of (29) and (30). The same methods used in this study have been employed by Saha et al., (28), Manukumar et al., (31), and Odekanyin and Akeredolu (32) to extract some bioactive proteins from *Cajanus cajan* seed coat, *Tinospora tomentosa* stem and *Pterocarpus osun* and *Bosquiea angolensis* seeds, respectively for the purpose of biological characterization.

Lectins are biological molecules present in almost all living organisms. Lectins are protein/glycoprotein that possess the ability to bind sugar moiety and agglutinate cells. Lectin plays significant roles in biological systems because of their...
therapeutic functions. Lectin activity was detected in the crude protein extract so also in the two fractions (25% and 60%). Crude protein extract and 25% fraction are not blood group specific but prefer rabbit blood erythrocytes while 60% fraction is rabbit blood specific. The lectin was also established to be mannose-binding lectin. Previously, mannose-binding lectins have been isolated and purified from *D. batatas* tuber (33) and *D. bulbifera* bulbil (34). In addition, lectin isolation, purification and biological characterization have been announced for other Dioscorea spp which include *D. preussii* (35), *D. mangenotiana* (36), *D. opposita* (37), *D. alata* (38), *D. japonica* (39) among others. Different biological and therapeutic activities such as antioxidant, insecticidal, anti-tumor, anti-proliferative and antiviral were established for the above-mentioned purified lectins. None of the lectins from Dioscorea spp purified up-to-date have been implicated in the inhibition of α-amylase and α-glucosidase or in any mechanism of diabetes mellitus management. Numerous reports on the lectins from other sources with anti-diabetic activity have been published (40 - 42).

Plant extracts have been used since time immemorial for the treatment and control of diseases. Based on the World Health Organization report of 2002, a higher percentage of the world

### Table 4. DPPH Radical Scavenging Activity of *D. bulbifera* Bulbil Protein Fractions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition ± SEM</th>
<th>IC$\text{}_{50}$ ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>0.625</td>
<td>-15.68±1.52</td>
<td>0.013±0.002</td>
</tr>
<tr>
<td></td>
<td>1.250</td>
<td>-10.30±0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.500</td>
<td>5.563±1.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.000</td>
<td>12.832±1.47</td>
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<td></td>
<td>10.00</td>
<td>39.381±1.92</td>
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</tr>
<tr>
<td></td>
<td>20.00</td>
<td>83.186±0.54</td>
<td></td>
</tr>
<tr>
<td>60%</td>
<td>156.25</td>
<td>11.44±1.83</td>
<td>3.404±0.01</td>
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<tr>
<td></td>
<td>312.50</td>
<td>14.54±0.98</td>
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</tr>
<tr>
<td></td>
<td>625.00</td>
<td>22.31±1.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1250.0</td>
<td>26.87±1.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2500.0</td>
<td>38.88±0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5000.0</td>
<td>39.57±0.81</td>
<td></td>
</tr>
<tr>
<td>25%</td>
<td>62.50</td>
<td>13.34±0.76</td>
<td>2.526±0.30</td>
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<td>125.0</td>
<td>14.48±0.76</td>
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<td>250.0</td>
<td>18.52±1.74</td>
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<td></td>
<td>500.0</td>
<td>24.65±1.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>31.42±1.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>41.21±2.06</td>
<td></td>
</tr>
</tbody>
</table>

![Figure 3. Hydroxyl Radical Scavenging Activity of *D.bulbifera* bulbil Protein Fractions.](image-url)
population is using traditional means to treat various diseases. This is because the tradomedical care is very cheap with rather no negative side effects when put side-by-side with the conventional medicine. Researchers have therefore focused on getting more scientific-based facts on the usage of plants and its products for the benefit of human health. Management of diabetes mellitus, a condition characterized by an increase in blood glucose level, is currently being treated with herbal extract, which is more effective, affordable, and with no or little adverse effects. At chronic stage, diabetes is associated with other diseases such as heart damage, eye blurring, kidney failure, stroke and other health challenges.

Among the mechanisms through which the plant-based drugs work in the management of diabetes are controlling hyperlipidemia, hyperglycemia and putting oxidative stress under control. Inhibition of carbohydrate-hydrolyzing enzymes (α-amylase and α-glucosidase) is currently employed as the main therapeutic means of managing diabetes mellitus conditions. These enzyme inhibitors are abundantly present in various parts of the plant. So far, many of the isolated, purified and tested inhibitors are obtained through extraction with organic solvent (15, 16, 43). Several reports on the efficacy of a variety of secondary metabolites as inhibitors of these enzymes are available (43, 44). Protein has also been reported to have inhibitory activity against α-amylase and α-glucosidase (45, 46). Studies conducted have also shown that protein extracted from plants plays a beneficial role in combating chronic diseases like

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition ± SEM</th>
<th>IC50 ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>7.81</td>
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<td>0.06±0.002</td>
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<td>15.63</td>
<td>16.61±2.63</td>
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<td>31.25</td>
<td>34.98±1.14</td>
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<td>62.50</td>
<td>52.86±0.75</td>
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<tr>
<td>125.0</td>
<td>86.86±0.58</td>
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<tr>
<td>60%</td>
<td>31.25</td>
<td>-17.45±2.37</td>
<td>13.93±4.88</td>
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<td>10.34±6.03</td>
<td>1.30±0.06</td>
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<td>2000</td>
<td>68.26±0.79</td>
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<table>
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<tr>
<th>Assays</th>
<th>Milligram ascorbic acid equivalent per gram of sample (mgAAE/g)</th>
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<tbody>
<tr>
<td>Ferric Reducing potential (FRAP)</td>
<td>1.935 ± 0.077</td>
</tr>
<tr>
<td>Total Antioxidant Capacity (TAC)</td>
<td>17.167 ± 0.785</td>
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cancer and diabetes particularly in the control of type 2 diabetes mellitus (15). These plant proteins are able to play the beneficial role as a result of the important bioactive compounds they contain, these bioactive components possess various biological properties such as antioxidant, analgesic and anti-inflammatory (47), anti-tumor (48), anti-diabetic and anti-microbial properties (49). Two-ammonium sulphate protein fractions obtained from *D. bulbifera* bulbil were effective in inhibiting α-amylase and α-glucosidase in-vitro. In vitro α-amylase and glucosidase inhibitory studies conducted by (15) revealed that *G. glauca* and *D. bulbifera* extracts had inhibitory activity against these enzymes. The percentage of inhibition depend on the type of organic solvent used for extraction and source of the enzyme employed for the assay and it ranges between 61.65% - 73.39% for α-amylase and 18.13% - 99.6% for α-glucosidase (15). In this study, 25% and 60% protein fractions of *D. bulbifera* bulbil were found to also display significant inhibition against α-amylase and α-glucosidase. Protein fractions of *D. bulbifera* bulbil (25% and 60%) show IC$_{50}$ values of 5 µg/ml and 6 µg/ml respectively, which makes it a better inhibitor of α-glucosidase than acarbose (IC$_{50}$= 28.11±2.7 µg/ml). Present study also revealed that 25% and 60% protein fractions of *D. bulbifera* bulbil are more potent inhibitors of α-amylase with IC50 values of 47.0 and 42.4 µg/ml respectively than acarbose. Storage protein of Dioscorea spp (Dioscorin) has not been reported to exhibit antidiabetic activity. Antidiabetic potentials of extracts obtained through organic solvent extraction from *D. alata*, *D. batatas* and *D. opposite* tubers have been reported (50-52). Excessive production of reactive oxygenated species or total failure of the endogenous antioxidants leads to oxidative stress. Oxidative stress has been reported as the main cause of numerous diseases such as ageing, cancer, neurodegenerative disorders, chronic inflammation, diabetes mellitus and atherosclerosis. Discovery of more potent antioxidant agents from plant sources that possess other therapeutic potentials will provide relief to the radical imbalance-mediated oxidative stress and associated diseases. This is a sequel to the various limitations of conventional drugs.

It is well established that the Dioscorea spp possess huge antioxidant potential confined in

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition ± SEM</th>
<th>IC$_{50}$ ± SEM</th>
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<tr>
<td>Ascorbic acid</td>
<td>1.563</td>
<td>-0.08±2.05</td>
<td>0.022±0.001</td>
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<td>3.125</td>
<td>8.10±1.01</td>
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<td></td>
<td>6.250</td>
<td>17.71±2.14</td>
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<td></td>
<td>12.50</td>
<td>41.94±0.48</td>
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<td>25.00</td>
<td>82.37±0.30</td>
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<tr>
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<td>50.00</td>
<td>84.63±0.38</td>
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</tr>
<tr>
<td>60%</td>
<td>62.50</td>
<td>37.11±1.50</td>
<td>1.036±0.0117</td>
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<td>125.0</td>
<td>56.52±1.26</td>
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<td>250.0</td>
<td>70.71±1.21</td>
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<td></td>
<td>500.0</td>
<td>76.75±0.92</td>
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<td>1000</td>
<td>79.22±0.53</td>
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<td></td>
<td>2000</td>
<td>81.89±0.87</td>
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<td>25%</td>
<td>62.50</td>
<td>-42.73±0.82</td>
<td>0.247±0.1151</td>
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<td>125.0</td>
<td>42.87±3.73</td>
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<td>46.43±4.80</td>
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<td>500.0</td>
<td>63.92±1.07</td>
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<tr>
<td></td>
<td>1000</td>
<td>65.36±0.05</td>
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<tr>
<td></td>
<td>2000</td>
<td>71.06±0.87</td>
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</table>

Table 7. Ferrous Ion-chelating Ability of *D. bulbifera* bulbil Protein Fractions.
the phytochemicals present in the plants (53). Only few reports have mentioned the antioxidant activity of Dioscorea spp proteins or its hydrolysates. The in-vitro antioxidant activity of protein fractions of D. bulbifera bulbil was determined using assay methods with different mechanisms, which include DPPH radical scavenging activity, Nitric oxide scavenging assay, hydroxyl radical scavenging assay, Total antioxidant capacity, Ferrous ion-chelating ability assay, and Ferric reducing antioxidant power.

DPPH radical scavenging assay is the simplest, most reliable, highly sensitive and acceptable antioxidant assay method coupled with its good reproducible results. DPPH is a nitrogen-containing compound that accepts electron or hydrogen atoms to become stable from antioxidants. The ability of antioxidants in the extract to scavenge the DPPH radical is the basis for the assay. This is noticed in the colour change from purple to yellow upon the formation of stable DPPH. Decrease in spectrophotometric reading at 517 nm allowed the evaluation of the antioxidant effect. The extract donates an electron or hydrogen atom to DPPH for it to become stable. Two protein fractions produced from D. bulbifera bulbil crude protein were able to bleach the DPPH colour that shows the capacity of the fractions to scavenge the free DPPH radical. Our findings compare favourably well with the available results of antioxidant capacity of a variety of Dioscorea spp (53). In the comprehensive review of the Dioscorea spp antioxidant potentials, various in-vitro chemical methods, in-vivo assays using rat and mouse model and cell cultures were employed. Different parts of the plants and a variety of organic solvent were reportedly used.

Lipid peroxidation and in vivo damage to biological molecules are due to hydroxyl radicals, an oxygen species with high reactivity produced by some metabolic pathways. Hydroxyl radical scavenging activity is used as a measure of antioxidant activity of biological extract or pure isolates that may function in food and health products. In-vitro generation of hydroxyl radical is through Fenton reaction using Fe2+/ascorbate/EDTA/H2O2 system. In the presence of a catalyst — transition metal ion (Fe2+) and hydroxyl radical at an acidic pH, malondialdehyde, which produces a coloured chromogen in the presence of thiobarbituric acid, is generated from the degradation of deoxyribose. Both 25% and 60% fractions exhibited better hydroxyl radical scavenging than ascorbic acid employed as standard. Sixty per cent (60%) protein fraction showed excellent scavenging activity, which was significantly different from 25% protein fraction and ascorbic acid. The result is in agreement with reports of (54) and (55). In their studies, Ghosh et al, (54) reported on the antioxidant activity of D. bulbifera bulb while Liu et al, (55) study centered on D. opposite rhizome peel and flesh. From the data presented, it could be suggested that the fractions render the process of Fe2+-dependent hydroxyl radical production inactive or poorly active, thereby reducing the effect of hydroxyl radical on biological systems.

Among the physiological roles of Nitric Oxide (NO) are acting as a vasodilator in the cardiovascular system, as an intracellular signal in several cellular pathways and playing a protective role against oxidative stress (56). Its plausible involvement in cancer treatment and SARS-COV-ID-19 has been reported (57). Also NO produced by macrophages protects the body against infection as an antimicrobial agent (58). Despite these significant roles, excessive increase in NO generation in intracellular space can lead to cytotoxic effects. Nitric oxide has therefore been linked as the root cause of many degenerative diseases. The clearance or removal of excess NO will definitely play high impact roles in management and treatment of such diseases. The Nitric Oxide scavenging ability of 25% protein fraction was evaluated by the decline in the absorbance reading at 546 nm, resulting from a nitric oxide generation decrease. The present study established the ability of 25% protein fraction to scavenge NO radical. This potential will assist in stopping further synthesis of a more reactive oxygen radical species that can increase the development of oxidative stress. Based on this 25% protein fraction may be suitable for the treatment and management of oxidative stress related ailments.

Ferric reducing antioxidant power assay permits the measurement of reduction of Fe3+-TPTZ to Fe2+-TPTZ by antioxidant compounds present in an extract under acidic condition. The
intense blue colour formed was absorbed maximally at 593 nm wavelength. The advantage of this antioxidant assay method is that it is fast, simple and gives reproducible results. It was first used to quantify ascorbic acid in serum and plasma (26, 58). Spectrophotometrically, the production of Fe$^{2+}$-TPTZ complex was monitored which indicates the reducing ability of protein fractions. 25% protein fraction has a significant potential to reduce Fe$^{3+}$ to Fe$^{2+}$ though lower than the standard used. Ghosh et al., (59) applied FRAP to determine the antioxidant activity of D. bulbifera bulb using Gallic acid equivalent antioxidant capacity to express their results. Moon and Shibamoto (60) suggested that the standard antioxidant for this assay must be water-soluble. Ascorbic acid equivalent used in the current study revealed that the results for 25% protein fraction is within the range reported in the literature for those that also used ascorbic acid equivalent (61, 62). A number of studies have stated that the reducing power of an extract or isolate may serve as an important pointer of potential antioxidant activity. Antioxidative activity has been suggested to be associated with reducing power.

The importance of heavy metals in the biological system cannot be over-emphasized. Among the events in the living system in which metals are involved are aiding enzyme catalysis, cell growth, biosynthesis of molecules, body immune system and cell proliferation and development (63). Presence of excess metal ions in different pathways is injurious to the biological systems. Transition metal ions, putting aside their goods, are also liable to cause peroxidation of unsaturated fatty acid and also contribute to the oxidative damage in some diseases such as Alzheimer’s and Parkinson’s diseases. The condition referred to as metal toxicity or poisoning can only be treated using Metal chelating therapy in which drugs like dimercapto-succinic acid, EDTA, deferoxamine and dimercaprol are prescribed for the patient. Metal chelating activity of the protein fractions was tested using ferrozine as a chelating agent. In this assay, decolorization of Fe$^{2+}$-ferrozine complex takes place due to the presence of chelating agents in the protein fractions of D. bulbifera bulbils. Colour reduction observed in the assay showed that the metal ion chelating activity of the protein fractions of D. bulbifera increases. The results revealed that the 25% protein fraction is five times better in chelating ferrous ions than 60% protein fraction. The result is in agreement with the study conducted by Narkhede and Jagtap, (64), it was observed that methanolic extracts of D. bulbifera bulbils and D. bulbifera root tuber showed a higher chelating activity than the other six different tubers tested.

Total antioxidant capacity (TAC) is based on the potential of an oxidative agent to reduce molybdenum (VI) to molybdenum (V). The assay is quantitative spectrophotometric techniques that measure both fat-soluble and water-soluble antioxidants present in a sample. There is formation of green phosphomolybdenum (V) complex without induction of free metal ions solution. It also gives a direct estimation of reducing power of antioxidants. The results revealed that 25% protein fraction has higher TAC expressed in ascorbic acid equivalent per gram of sample than 60% protein fraction. This showed that the 25% fraction contains molecules that can compete with ferrozine for ferrous ions thereby preventing the formation of Fe$^{2+}$-ferrozine complex. This fraction can be of great therapeutic importance especially in treatment of disease conditions called Thalassemia or other anemia ailments. The excess iron in the body is neutralized using metal chelating therapy in which the 25 % fraction can replace the expensive synthetic drugs that also have some side effects. Prieto et al, (25) stated that this method aside its usage to analyze the antioxidant contribution of specific food components, it can also be employed in analyzing changes in plasma antioxidant activity related to oxidative stress and in understanding the structure activity relationship of pure antioxidant molecules.

5. Conclusion

The data presented clearly showed and established the inhibitory potential of 25% and 60% protein fractions against the activity of carbohydrate-hydrolyzing enzymes (α-amylase and α-glucosidase). Their inhibitory action was better than that of standard drug (acarbose) used in this study. The fractions were also found to display antioxidative prowess by clearing the free radicals
Antioxidant and antidiabetic potential Dioscorea bulbifera bulbil protein

from solution. Ability to chelate metal ions was also established coupled with potentials to reduce ferric ions to ferrous ions. These properties could help in mitigating the development of oxidative stress, the sole cause of several biological damages that lead to numerous ailments. The fractions can be considered for possible development as a promising therapeutics agent for the treatment of diabetes and other oxidative stress-related diseases. Therefore, the ineffectiveness of 90% protein fraction in the manifestation of anti-amylase and anti-glucosidase activities may be due to the presence of high molecular weight proteins that could possibly block the antioxidant activity of lower molecular weight proteins. The fractions can therefore be subjected to further studies like in-vivo animal model experiment and purification of the lectin and other possible active components in the fractions.

Conflict of Interest

The authors declare no conflict of interest.

References


13. Subasini U, Thenmozhi S, Sathyamurthy


30. Libra MA, Gonnety JT, Ahl AP, Dabonne S, Ahiho ED, Kouame LP. Physicochemical changes in bulbils of two cultivars of Dioscorea

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**Note:** The text above contains a variety of scientific references and studies related to pharmaceutical sciences, emphasizing the use of medicinal plants and their constituents for various medical applications. The references range from 1982 to 2013, indicating a comprehensive review of the research conducted during this period. The studies cover a wide range of topics, from pharmacognostic investigations to the evaluation of plant extracts for their potential health benefits, particularly in diabetes and antioxidant activities.