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ORIGINAL ARTICLE

Hemagglutinin from the Root Tuber of *Dioscorea preussii* Pax

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ABSTRACT

Objectives: In this study, *Dioscorea preussii* root tuber hemagglutinin was purified and its physicochemical properties were determined. The antioxidative and anti-hemolytic activities of the hemagglutinin were also investigated.

Materials and Methods: The hemagglutinating assay was used to detect the presence of lectin in the phosphate-buffered saline extract of the *D. preussii* root tuber. The lectin was purified using ammonium sulfate fractionation and molecular sieve chromatography. The optimum pH and temperature were determined. In addition, antioxidant activity was assessed using 2,2 diphenylpicrylhydrazyl (DPPH) radical scavenging, metal chelating, ferric reducing antioxidant power (FRAP), and lipid peroxidation inhibition assays. Red blood cells subjected to oxidative damage caused by H_2O_2 were employed to evaluate their antihemolytic ability.

Results: Starch inhibited hemagglutinin activity. *Dioscorea preussii* hemagglutinin (DPH) maintained full hemagglutinating activity from 30 °C to 60 °C and pH 5-13. Ethylene diamine tetraacetic acid did not affect the hemagglutinating activity of hemagglutinin. All denaturing agents (Guanidine-HCl, urea, and β-mercaptoethanol) reduced the hemagglutinating activity of the hemagglutinin to different degrees. The hemagglutinin scavenged the DPPH radical and chelated iron metal with half maximum inhibitory concentration (IC_{50}) of 0.727 ± 0.035 mg/mL and 0.583 ± 0.078 mg/mL, respectively, while the FRAP assay showed that it contained 76 mg of ascorbic acid equivalent per gram of the purified hemagglutinin. In the absence of hemolytic agents and at lower concentration tested, hemagglutinin showed positive membrane integrity protection.

Conclusion: This study provides information on the antioxidant properties of *D. preussii* root tuber hemagglutinin as well as its cell membrane protective ability. The lectin is a starch-binding, which makes it a novel lectin.

Keywords: *Dioscorea preussii*, hemagglutinin, antioxidant, anti-hemolytic, lectin

INTRODUCTION

Dioscorea preussii as a genus belongs to the family *Dioscoreaceae*, which is the most recognized among all families under the order *Dioscoreae*. This genus is primarily found in West Africa, tropical America, and Southeast Asia. *Dioscorea* spp. (*Dioscoreaceae*), which are commonly referred to as Yams in English, are tuberous plants with stems that climb and twine either to the right or left. The Yorubas, Igbos, and Hausas in Nigeria refer to yam as is, ji, and dopa, respectively.[1] Food and health issues continue to be the main challenges in developing countries. Many wild crops are still unexplored; unfortunately, great medicinal and nutritional solutions that the world is looking for might just be locked up in some of these crops. Therefore, scientific research must address these problems robustly by looking for other food and medicinal sources.[2]

There are many wild medicinal and edible products that nature has provided like tubers, stems, nuts, roots, fruits, and flowers.[3,4] Apart from the significant roles of yam in fighting the scarcity of food, literature has shown that *Dioscorea* species possess biological activities like antiproliferative, antioxidant, androgenic, lectin, antimicrobial, and immunomodulatory activities, among others.[5-7]

Very little is known about *D. preussii* Pax, as extremely few studies[8,9] have been conducted on this wild plant. It is a climber whose stem twines left-handed, with the ability to go up to 24 m from its narrow cylindrical tuber. The tuber, which is usually deeply buried, can be as long as 40 cm, with horizontal branches that can also be as long as 30 cm long. Tuberculosis is mostly eaten in some places during famine.[10,11] In Nigeria, the tuber is

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known as ainyelo, igiruesi, and esuru-igbo among the Igbo, Edo, and Yoruba people, respectively.[10,12] The extract of *D. preussii* has been reported to exhibit *in vitro* cytotoxicity, antileishmanial, and antifungal activities.[13] Tabopda et al.[8] isolated and characterized three important steroidal saponins from this tuber.

During cellular metabolism, which is a form of normal cellular activity, free radicals are produced. These free radicals can simply be described as atoms or molecules containing a single electron or more in their outer orbits, making these atoms or molecules unstable and highly reactive. These reactive species at low or moderate levels perform beneficial roles in immunity, redox regulation, cellular signaling pathways and in mitogenic response.[14] At higher concentrations, reactive oxygen species (ROS) cause oxidative stress, whereas reactive nitrogen species cause nitrosative stress, which destroys biomolecules. Biomolecules such as deoxyribonucleic acid (DNA), lipids, and proteins could have their integrity damaged by excess ROS, thereby causing an upsurge in the imbalance in oxidant-antioxidant levels found in different diseases in humans such as cardiovascular diseases, diabetes mellitus, cataracts, rheumatoid arthritis and others.[14]

To prevent the destructive effects of free radicals, the human body has devised different mechanisms to combat them through certain agents known as antioxidants.[15] Antioxidants are molecules that neutralize free radicals to prevent their cellular damage. They do this by donating electron(s) to reactive species, chelating metals, hydrogen donation, enzyme inhibition, and peroxide decomposition.[15,16]

Hemolysis is the rupturing of the erythrocytes.[17] Red blood cells are known to possess abundant polyunsaturated fatty acids, high oxygen, hemoglobin, and membrane proteins. Because erythrocytes are more exposed to oxygen than any other cell or tissue in the body, they are more vulnerable to oxidative destruction; thus, they are frequently and widely used in the study of oxidative damage in membranes. Moreover, although hemoglobins present in erythrocytes are strong catalysts that can lead to the initiation of lipid peroxidation, the invasion of the membranes of erythrocytes by peroxidases can also result in cell hemolysis.[18,19] This is resulted in peroxidation of unsaturated membrane fatty acids leads to disruption of the usual organization of membrane lipids, membrane pore formation and water permeability alteration, eventually damaging the membrane structure leading to hemolysis.[20] Because of its relatively high stability and diffusion of hydrogen peroxide, it is considered an excellent oxidant model for investigating both the hemolytic and anti-hemolytic activities of various samples.[19] Different natural substances have been proposed as therapeutic agents to prevent hemolysis, some of which have anti-hemolytic potential linked to their free radical scavenging activity.[21,22]

Lectins are ubiquitous (glyco) proteins that possess at least one binding site for carbohydrate or their derivatives, have no catalytic function, and are also of non-immunoglobulin origin.[23] All of these proteins were initially referred to as hemagglutinin, a term currently used only for those whose sugar specificity is unknown or unknown. Plants are a great source of lectins, and they are also the major source from which lectins that are analyzed are isolated. Lectins are known to have many biological properties including antifungal, antitumor, antibacterial, antiviral, germicidal, and insecticidal activities.[24] Lectins are also known to perform various roles including storage, transport, signaling,[25,26] cell recognition, cell migration, endocytosis, complement activation, cell adhesion, intracellular translocation processes, apoptosis activation, cell signaling, immune regulation, and defense against pathogens.[25,27]

Under the condition of severe redox imbalance, erythrocytes become vulnerable and cellular defense does not offer full defense against the attack of reactive and free radicals, which could lead to oxidative damage-related diseases such as cardiovascular disease. Recently hemagglutinin, which possesses antioxidant activity, has been reported[28,29] from plant sources and protects erythrocytes from hemolysis. The literature search indicated that there is a need for more plant-derived proteins with such activity. Therefore, identifying hemagglutinin with antioxidant potential and protective effects on erythrocytes that have little or no side effects will be of great benefit. This study led to the purification of a novel hemagglutinin from the root tuber of *D. preussii* and the determination of its physicochemical properties. This study also investigated the antioxidant and anti-hemolytic activities of hemagglutinin with a view to exploring its therapeutic potential.

MATERIALS AND METHODS

All experiments, or otherwise stated, were carried out in the Protein Science Laboratory, Biochemistry, and Molecular Biology Department, Obafemi Awolowo University, Ile-Ife, Nigeria.

Blood collection

Different blood groups under the ABO blood group classification were drawn from apparently healthy human donors into heparinized bottles. Erythrocytes were also obtained from healthy rabbits purchased from Teaching and Research Farm, Obafemi Awolowo University, Ile-Ife, Nigeria.

Preparation of D. preussii crude extracts

Root tubers of *D. preussii* were collected from a farm in Obafemi Awolowo University, Ile-Ife, Nigeria. The root tubers of *D. preussii* were thoroughly washed to remove sand particles and then peeled. The peeled tubers were minced into very small pieces and ground into a fine paste. Ground tubers were extracted at 4 °C overnight in phosphate-buffered saline (PBS, pH 7.2) at a ratio of 1:10 *w/v*. The mixture was sieved with cheesecloth; the filtrate was later centrifuged at 4 °C and 10,000 rpm for 20 minutes using a cold centrifuge (Centurion Scientific LTD. 8880, R-Series). The resulting supernatant is hereafter termed the crude extract.

Glutaraldehyde fixation in erythrocytes

To fix the erythrocytes, blood samples were collected from humans and animals into heparinized bottles. The samples were immediately centrifuged at 3,000 rpm using a centrifuge

(Hospibrand 0502-1) for 15 minutes 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 fixation, the mixture was centrifuged for 5 minutes at 3000 rpm to collect the fixed erythrocytes. The fixed erythrocytes were extensively washed with PBS to remove glutaraldehyde. Two percent of the erythrocytes were prepared in PBS containing 0.02% sodium azide. This was stored in a refrigerator for further use.

Hemagglutinating assay

The presence of lectin in the crude extracts of *D. preussii* and its various fractions was determined using a modified hemagglutinating assay procedure involving Odekanyin and Kuku.[30] The assay was performed in a 96-well U-shaped microtiter plate. PBS (100 µL) was sequentially pipetted into all wells of the microtiter plate. After this, 100 µL of the crude extract or any fraction was added to the first well in the first row, and the mixture was serially diluted up to the 24th well (2 rows). Fifty microliters of the fixed erythrocyte suspension (50 µL) were pipetted into each well. The plate was left undisturbed for 2 hours on a laboratory bench and then observed for any visible hemagglutination. The control experiment did not extract any fractions. Haemagglutination titer unit was taken as the highest dilution reciprocal of the crude extract or any of the fractions producing visible haemagglutination. Specific activity is the haemagglutination titer unit number per mg protein expressed as hemagglutinating units/mg.

Sugar specificity test

The sugar specificity of *D. preussii* hemagglutinin (DPH) was determined by comparing the abilities of different sugars to inhibit the hemagglutinating activity of the hemagglutinin. First, the serial dilution of the hemagglutinin was carried out as described in hemagglutinating assay section until the last dilution at which hemagglutination was observed. Sugar solution (0.2 M, 50 µL) was added to each well, whereas PBS-supplemented sugar solution was added to the control wells. The microtiter plate was then incubated at room temperature for 2 hours. The erythrocyte suspension (50 µL) was added to each well. The plate was left undisturbed for 2 hours on a laboratory bench and then observed for any visible hemagglutination. The tested sugars were glucosamine HCl, lactose, maltose, sorbose, starch, mannitol, galactose, N-acetyl-D-glucosamine, xylose, arabinose, glucose, dulcitol, α-D-methyl glucopyranoside, and 2-deoxy-D-glucose.

Protein concentration determination

Lowry method[31] of protein concentration determination was adopted to determine the total protein concentration of the crude extract and other fractions. Bovine serum albumin was used as the standard.

Purification of lectin

Ammonium sulfate fractionation

Solid ammonium sulfate (16.4 g/100 mL) was mixed with a known volume of the crude extract with gentle stirring to reach a solution containing 30% ammonium sulfate saturation. After 24 hours, the mixture was centrifuged at 4,000 rpm for 10 minutes to collect the precipitate. This precipitate represents the 30% ammonium sulfate fraction. The ammonium sulfate saturation of the supernatant was then increased to 60% through slow addition with gentle stirring of solid ammonium sulfate (18.1 g/100 mL). The precipitate was also collected and represented a 60% ammonium sulfate precipitate fraction. Again, the ammonium sulfate saturation of the supernatant was increased to 90% by slowly adding solid ammonium sulfate (20.1 g/100 mL). The precipitate representing 90% of the fraction was also collected. All precipitates were separately dialyzed against PBS exhaustively, and the dialysate of each fraction was tested for hemagglutinating activity. The dialysates were later stored in a deep freezer (below -4 °C)

Gel filtration on Sephadex G-100

Approximately 15 g of Sephadex G-100 was preswollen in PBS (200 mL) at room temperature for 72 hours. A column (2.5 x 40 cm) was packed with the preswollen resin and equilibrated with PBS (500 mL, 25 mM, pH 7.2). 5 mL of *D. preussii* ammonium sulfate dialysate was layered on the packed column, the same buffer (PBS) used for eluting proteins while collecting 5 mL fractions at a 20 mL/h flow rate. The hemagglutinating activity and protein concentration of these fractions were evaluated. Fractions with high hemagglutinating activity were pooled together, dialyzed exhaustively in phosphate buffer (pH 7.2, 0.01 M), and finally freeze-dried for further use.

Physicochemical characterization of lectin

Temperature effect on hemagglutinating activity

The purified hemagglutinin was incubated at various temperatures (30 °C-100 °C) for 60 minutes. Aliquots of the hemagglutinin at each temperature at 30 and 60 min and thereafter subjected to hemagglutinating assay.

pH effect on hemagglutinating activity

An Aliquot of the purified hemagglutinin was incubated for 1 hour with various buffers with different pH values to evaluate the effect of pH on the hemagglutinating activity of purified hemagglutinin. The same concentration (0.2 M) of the different buffers was used, and their pH ranges were as follows: Glycine-HCl buffer (pH 1-3), Acetate buffer (pH 4-6); Tris-HCl buffer (pH 7-8) and Glycine-NaOH buffer (pH 9-13). The incubated aliquots of hemagglutinin were subjected to a hemagglutinating assay after incubation to determine residual activity.

Denaturants effect on hemagglutinating activity

An aliquot of the purified hemagglutinin was incubated for 6 hours in 2-8 M concentrations of different denaturants (Guanidine HCl, urea and Mercaptoethanol) to determine the effects of the denaturants on the lectin activity of the purified hemagglutinin. The hemagglutinin incubated in PBS served as the control. The incubated mixtures were then assayed for hemagglutinating activity before and after dialyzing against PBS.

Chelating agents' effect on hemagglutinating activity

Purified hemagglutinin was dialyzed against two different concentrations of ethylene diamine tetraacetic acid (EDTA) (10 mM and 50 mM) separately for approximately 24 hours at 4 °C to determine the effect of EDTA on DPH. Subsequently, it was assayed for hemagglutinating activity.

Antioxidants assays

Scavenging of DPPH radicals

The purified hemagglutinin was assessed for its ability to scavenge DPPH radicals according to the method of Huh and Han.[32] Three-tenths millimolar DPPH in methanol (1 mL) was mixed with varying concentrations of the hemagglutinin (sample)/ascorbic acid (standard), after which the mixture was incubated for half an hour in a dark cupboard. Absorbance was measured at 517 nm using a Biobase ultraviolet-visible spectrophotometer BK-D5 series compared with a control in which the sample/standard was substituted with methanol.

The following formula was used to calculate the percentage inhibition:

$$\text{Percentage DPPH}^{\bullet}\text{ inhibition} = \frac{(A_{control}\text{-}A_{sample})}{A_{control}} \times 100$$

Here, $A_{control}$ denotes the absorbance of the control, and A_{sample} denotes the absorbance of the tested samples.

Ferric reducing antioxidant power (FRAP) assay

To prepare the FRAP reagent according to Benzie and Strain,[33] 300 mM acetate buffer (pH 3.6), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ, 10 mM, in 40 mM HCl), and 20 mM $FeCl.6H_2O$ at a ratio of 10:1:1 were mixed. Varying concentrations of ascorbic acid (50 µL, 0.02-0.1 mg/mL) or hemagglutinin (50 µL of 0.1 mg/mL) were added to FRAP (1 mL). Ten minutes after mixing, absorbance was measured at 593 nm against a blank reagent. The samples were protected from direct sunlight. The ascorbic acid equivalent (AAE) of the sample was estimated. The equivalent concentration was used to express the reducing power.

Lipid peroxidation inhibition assay

This assay was performed in accordance with the previously described procedure of Masao et al.[34] Butylated hydroxyltoluene (BHT) was used as the positive control. Egg homogenate (0.25 mL of 10%) was mixed with 0.1 mL Tris-HCl buffer (150 mM, pH 7.2), 0.05 mL of 1 % (*w/v*) ascorbic acid, 0.05 mL of 0.07 M $FeSO_4$, and various concentrations of the sample/standard, after which the mixture was incubated at 37 °C for an hour. After that, 0.67% thiobarbituric acid (TBA, 2.0 mL) was added. The mixtures were incubated for 30 minutes at 100 °C, allowed to cool, with the subsequent addition of butane-1-ol (2.0 mL), and centrifuged for 10 minutes at 3000 rpm. The absorbance of the supernatant was measured with the reagent blank as a reference at 532 nm.

The percentage inhibition of lipid peroxidation was estimated using the equation below:

$$\text{Lipid peroxidation inhibition} = \frac{A - B}{A} \times 100$$

Where A represents the absorbance of the control (without test sample), B represents the absorbance of the tested samples.

Metal-chelating activity assay

The ability of the DPH to form a complex with iron was determined using a metal chelating assay. The method proposed by Singh and Rajini[35] was used with minor modifications. Stock solutions of 2 mM $FeSO_4{\cdot}7H_2O$ and 5 mM ferrozine were diluted 20 times. $FeSO{\cdot}7H_2O$ (1 mL) was combined with various concentrations of hemagglutinin (1 mL)/EDTA (1 mL). After incubating for 5 minutes, ferrozine (1 mL) was added to start the reaction, and the reaction mixture was vortexed and incubated for another 10 minutes. The absorbance of the solution was then measured at 562 nm spectrophotometrically. The formula below was used to calculate the percentage inhibition of the formation of ferrozine-Fe^{2+}:

$$\text{Percentage Chelation} = \frac{(A_{control}\text{-}A_{sample})}{A_{control}} \times 100$$

Anti-hemolytic activity assay

The human blood group O-positive specimen used in this assay was first prepared before use. The blood drawn from a healthy donor was washed five times with PBS *via* centrifugation. Five percent (5%) of the washed erythrocytes were then prepared in PBS. Ebrahimzadeh et al.[36] procedure was adopted with little modification to determine the anti-hemolytic activity. Fifty microliters of different concentrations of hemagglutinin/ standard (ascorbic acid) were added to the erythrocyte suspension in PBS (5%, 100 µL). After 30 minutes of incubation, 100 µL of H_2O_2 (1.77 M) was added to the mixture. Subsequently, incubation for 3 hours at 37 °C followed by gentle shaking of the mixture. After incubation, the reaction mixture was centrifuged for 10 minutes at 2500 rpm. The supernatant was collected, and the absorbance was read at 540 nm to estimate the amount of hemoglobin released. The erythrocytes were also treated as described above, but without the hemagglutinin to obtain complete hemolysis taken as 100%.

To evaluate hemolysis induced by the sample (hemagglutinin), red blood cells were pre-incubated with the sample (hemagglutinin, 50 µL), after which the amount of hemoglobin released was determined. The percentage of hemolysis was calculated using the following formula:

The following formula was used to calculate the percentage of anti-hemolysis:

$$\text{Percentage anti-hemolysis} = \frac{A_2 - A_1}{A_2} \times 100$$

$$\text{Percentage hemolysis} = \frac{A_1}{A_2} \times 100$$

Hemolysis due to H_2O_2 (100 µL) was taken as 100% hemolysis. A_1 represents the absorbance of the tested samples. A_2 represents absorbance at 100% hemolysis.

Statistical analysis

The experiments were performed in triplicate, and the results were expressed as mean ± standard error of the mean and were analyzed using one-way analysis of variance for multiple measurements. GraphPad Prism statistical software version 7.0 (San Diego, California, 92108, United States) was used for all statistical analyses.

Ethical approval

The study approved by the Obafemi Awolowo University Departmental Health Research Ethic Committee (approval number: OAUBCH/HREC/2024/005, date: l4.08.2024).

RESULTS

The crude extract of *D. preussii* tubers agglutinated all the human blood group (ABO) and rabbit erythrocytes non-specifically. It displayed equal preference for all the erythrocytes tested (Table 1).

The binding specificity of *D. preussii* hemagglutinin to carbohydrates was investigated by reacting it with different sugars, none of them was able to completely inhibit the hemagglutinating activity of the haemagglutinin except a polysaccaharide, starch. The hemagglutinating activity was slightly inhibited by glucose, lactose, mannitol and ducitol, while fructose, galactose, maltose, arabinose, and methyl α-D-glucopyranoside had no effect on the hemagglutinating activity. Xylose and N-acetylglucosamine activated the hemagglutinating activity of the haemagglutinin.

The dialysate of the 60% ammonium sulphate precipitate was found to have higher activity than the dialysates of the 30% and 90% ammonium sulphate precipitates (Table 2).

Table 1. Blood group specificity of *Dioscorea preussii* crude extract

Blood type (Erythrocyte)		Hemagglutinating titer
Human blood group	A	2^{11}
	B	2^{11}
	O	2^{11}
	AB	2^{11}
Rabbit		2^{11}

Table 2. Hemagglutinating activities of dialysates with 30%, 60%, and 90% ammonium sulfate precipitates of *Dioscorea preussii*

Dialysates	Hemagglutinating titer
30%	2^6
60%	2^{10}
90%	2^5

The three ammonium sulphate fractions (30%, 60% and 90%) were subjected to gel filtration on Sephadex G-100. Sixty percent (60%) ammonium sulphate precipitate dialysate gave one major protein peak with 2 minor peaks. The haemagglutinating activity was found to reside in the major protein peak. The fractions of the activity peak were pooled. The specific activity and purification fold were 118.5 and 13.48 respectively (Table 3). The typical chromatogram of gel filtration on Sephadex G-100 of dialysate of 60% ammonium sulphate precipitate is shown in Figure 1A. The 30% and 90% ammonium sulphate precipitate dialysate were also gel filtered. Typical chromatogram of the gel filtration of 30% and 90% ammonium sulphate precipitate dialysates are shown in Figures 1B and 1C respectively. The active fractions were pooled and had insignificant hemagglutinating activity.

The hemagglutinating activity of *D. preussii* hemagglutinin was found to be completely stable up to 60 °C, there was a slight reduction of its activity at 70-90 °C, but totally lost its activity at 100 °C (Figure 2A).

The optimum pH of the hemagglutinin was between pH 5 and pH 13, which cover the neutral and basic pH range. It was noticed that the activity at the acidic pH range increases with increase in pH up to pH 5 (Figure 2B).

EDTA at two different concentrations (10 mM and 50 mM) had no effect on the hemagglutinating activity of the hemagglutinin after prolong dialysis.

Result revealed that all the denaturing agents were able to reduce the hemagglutinating activity of the hemagglutinin to different degrees as shown in Figure 2C. Mercaptoethanol at 1M to 7M was found to reduce the activity of the haemagglutinin to 50%, while 8M further reduced the activity to 25%. The hemagglutinating activity of *D. preussii* hemagglutinin was also found to have been reduced by Guanidine HCl at 4M and 5M to 50%, which was further reduced to 25% at 6M to 8M. Urea was found to reduce the hemagglutinating activity of the haemagglutinin at 8M by 50%.

Figure 3A shows the DPPH radical scavenging activity of *D. preussii* hemagglutinin. The haemagglutinin was found to possess DPPH radical scavenging activity in a concentration dependent manner, with an IC_{50} of 0.727 ± 0.035 mg/mL which was lower in activity than that of the standard (Ascorbic acid) having an IC_{50} of 0.022 ± 0.001 mg/mL.

The result of the metal chelating assay revealed that *D. preussii* was able to chelate iron in a dose dependent manner (Figure 3B). The IC_{50} of the haemagglutinin was found to be 0.583 ± 0.078 mg/mL which was lower in activity when compared with that of the standard (EDTA) possessing an IC_{50} of 0.041 ± 0.006 mg/mL.

D. preussii hemagglutinin unlike the standard (Butylated hydroxyltoluene) possessed no anti-lipid peroxidation activity. The level of lipid peroxidation was however found to increase as the concentration of the hemagglutinin increased, while the opposite was observed for the standard, that is, as the concentration of BHT increased, the percentage inhibition of lipid peroxidation also increased (Figure 3C).

D. preussii haemagglutinin exhibited high antihemolytic activity in the absence of hemolytic agent. Up to 90% antihemolytic activity was recorded and the activity was concentration related. The lower concentration of the hemagglutinin also protected the cell membrane integrity against oxidative stress caused by hydrogen peroxide.

DISCUSSION

In this study, the crude protein extract obtained from the root tubers of *D. preussii* contained hemagglutinin. The isolated and purified hemagglutinin was termed DPH. DPH hemagglutinating activity was non-specific against all types of red blood cells, as it agglutinated all the human blood group (ABO) and rabbit erythrocytes. This result showed that lectin possessed blood

Table 3. Purification table of hemagglutinin from *Dioscorea preussii* tuber

Fractions	Total protein level (mg)	Total activity (HU)	Specific activity (HU/mg)	Yield %	Purification fold
Crude extract	233.10	2048	8.79	100	1
Ammonium Sulfate precipitates dialysate (60%)	33.50	1024	30.57	50	3.48
Gel filtration (Sephadex G-100)	2.16	256	118.5	12.5	13.48

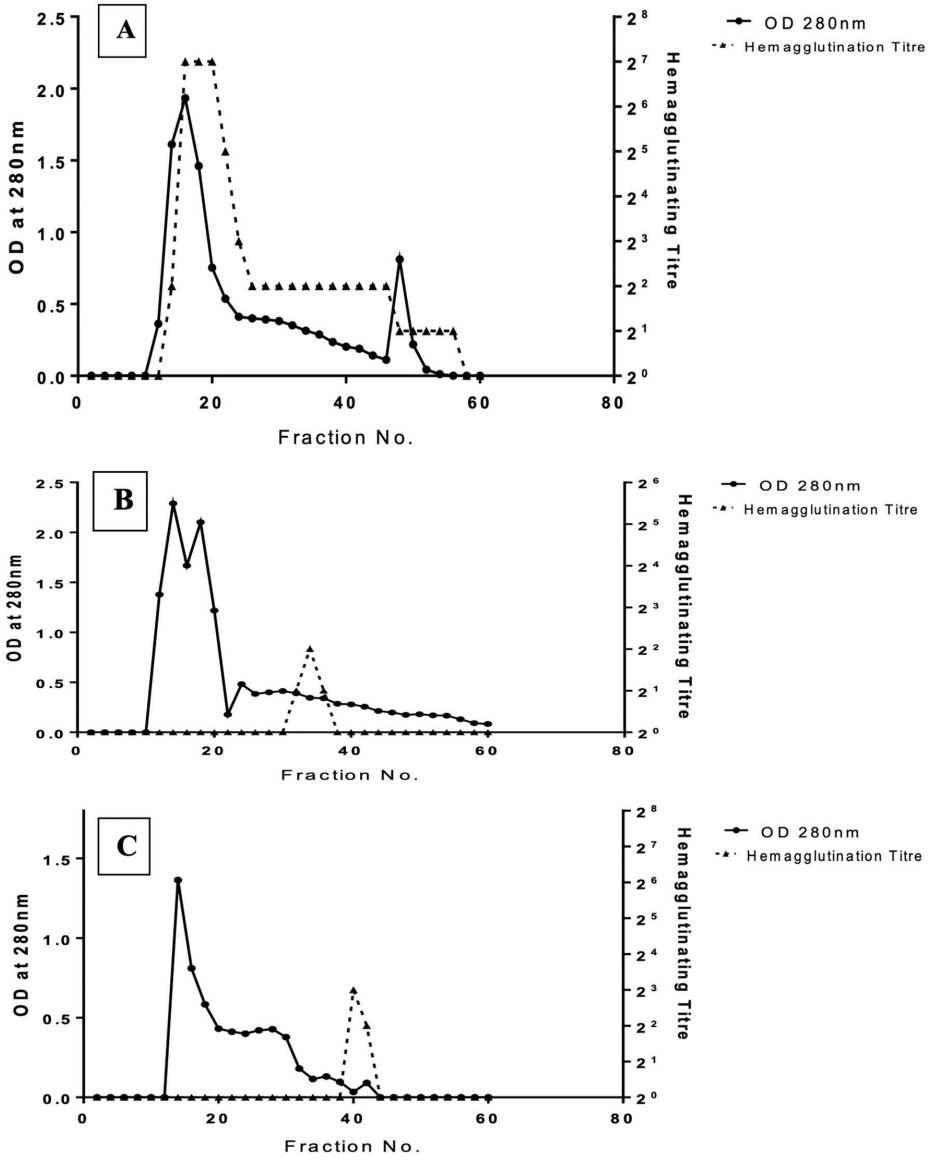

Figure 1. Gel Filtration Chromatogram of the Dialysate of the 60% (A), 30% (B), and 90% (C) $(NH_4)_2SO_4$ precipitate of *Dioscorea preussii* crude extract on Sephadex G-100

group specificity that was different from that of most tuber lectins. Tuber lectins are majorly rabbit erythrocytes specific. Tuber lectins from *Arisaema utile* Hook. F. Scott,[37] *Caladium bicolor* (Aiton) Vent.,[38] and *Dioscorea bulbifera* L.[39] have all been reported to agglutinate rabbit erythrocytes but are unreactive to human ABO blood group erythrocytes. Recently, a contrary report was published by Akinyoola et al.[40] Their report showed that the tuber lectin of *Dioscorea mangenotiana* J. Miège agglutinated all the human blood group (ABO) and rabbit erythrocytes non-specifically. These findings are consistent with the results reported in this study. In support of this, Sharma et al.[41] published isolation and purification of *Adenia hondala* (Gaertn.) de Wilde tuber lectin, a human blood group non-specific antibody that agglutinated rabbit erythrocytes.

A hapten inhibition assay, which determines the ability of sugars to inhibit the hemagglutinating activity of *D. preussii* tuber hemagglutinin, was performed. Different sugars including monosaccharides, disaccharides, and their derivatives along

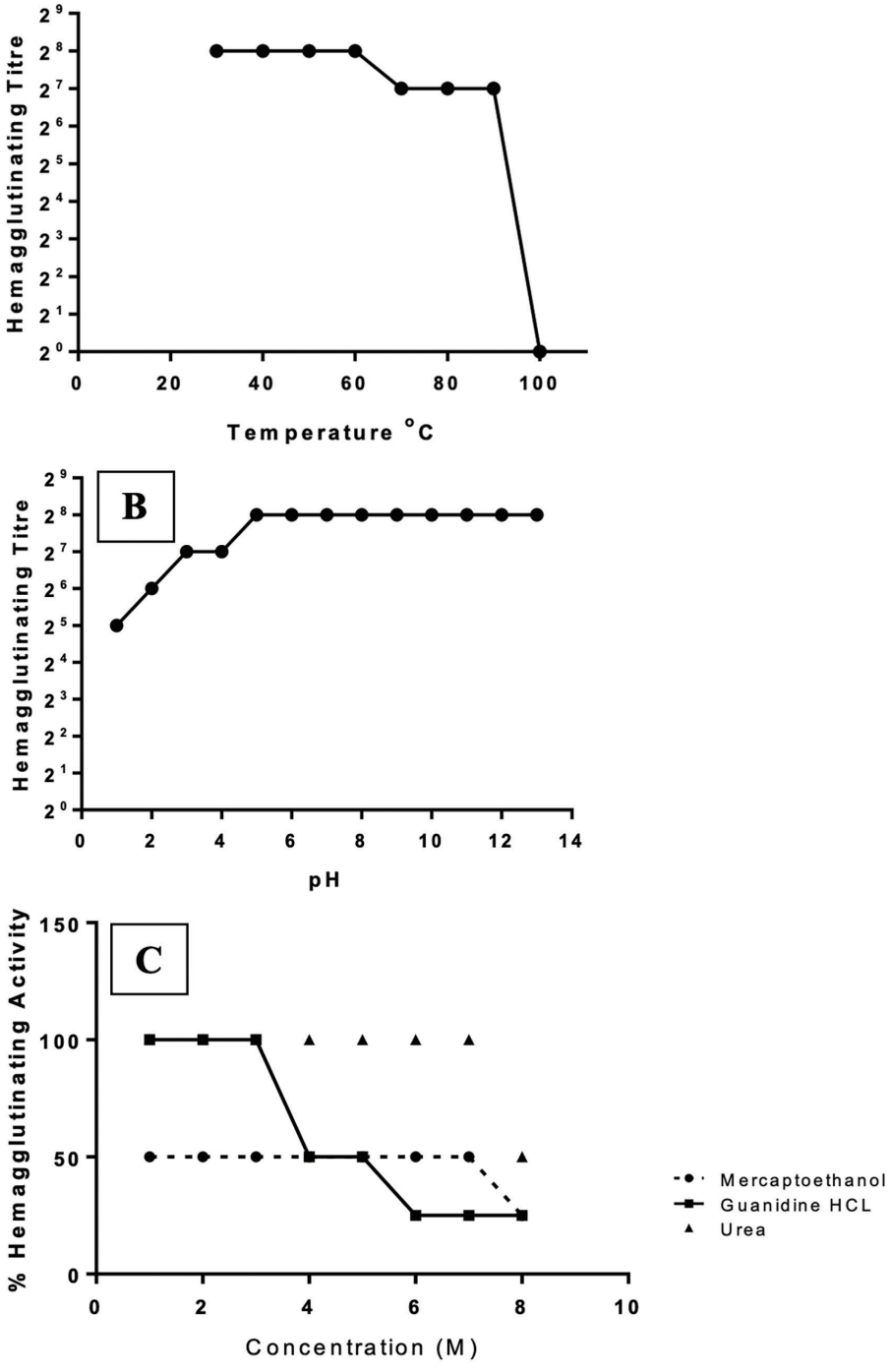

Figure 2. Effects of temperature, pH, and denaturing agents on DPH hemagglutinating activity
DPH: *Dioscorea preussii* hemagglutinin

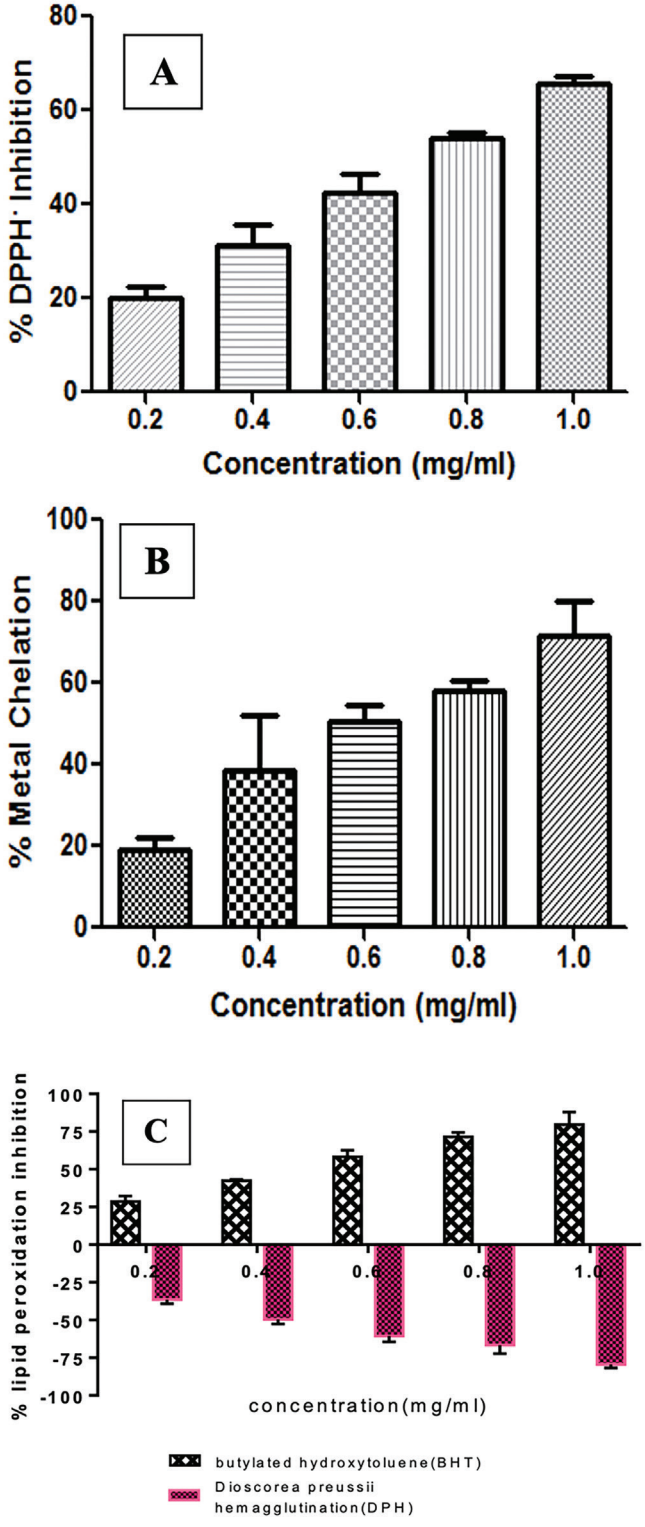

Figure 3. Antioxidant activity of *Dioscorea preussii* hemagglutinin. A) DPPH radical-scavenging activity, B) metal-chelating activity, C) anti-lipid peroxidation activity

DPPH: 2,2 diphenylpicrylhydrazyl

with some polysaccharides were used for this study, but none of the sugars inhibited the hemagglutinating activity of hemagglutinin except starch. This is compatible with the result of Sharma et al.[39] It was reported that all of the tested simple sugar derivatives and the simple sugar themselves did not inhibit the hemagglutinating activity of the aerial tuber of *D. bulbifera* lectin. Pereira et al.[42] reported that all the tested sample sugars did not inhibit the hemagglutinating activity of *Colocasia esculenta* lectin. Contrary to this, Akinyoola et al.[40] reported that glucose and N-acetylglucosamine inhibited *D. mangenotiana* tuber lectin activity. Galactose was also found to inhibit the activity of *Dioscorea opposita* tuber lectin.[43] It was discovered in the current study that a polysaccharide of plant origin (starch) inhibited the hemagglutinating activity of *D. preussii* tuber hemagglutinin.

We subjected the crude lectin extract from the root tubers of *D. preussii* to salt fractionation using ammonium sulfate. The dialysate of the 60% ammonium sulfate precipitate was found to have higher hemagglutinating activity than the dialysates of the 30% and 90% ammonium sulfate precipitates (Table 2). Therefore, it was purified and used for further studies. Also, 60% ammonium sulfate precipitate dialysate gave one major protein peak with 2 minor peaks when gel-filtered on a Sephadex G-100 column. The hemagglutinating activity was found to reside in the major protein peak. The fractions of activity peaks were pooled. The specific activities and fold purifications were 118.5 and 13.48, respectively (Table 3). The typical chromatogram of gel filtration on Sephadex G-100 of dialysate with 60% ammonium sulfate precipitate is shown in Figure 1A. Dialysates with 30% and 90% ammonium sulfate precipitate were also gel-filtered. The active fractions were pooled and had insignificant hemagglutinating activity (Figure 1B and 1C). Purity of protein is initially determined by the specific activity of the protein and later by electrophoresis. Specific activity is the amount or activity of such protein per milligram of whole protein in the sample. The higher the specific activity the better the purity of the protein. Pure protein is obtainable through the usage of chromatographic techniques. A single step or combination of different chromatographic methods can be adopted for purification procedure. In this study, a single step chromatographic technique (gel filtration) was adopted. The process produced hemagglutinin with specific activity of 118.5 HU/mg and purification fold of 13.48. Similar procedure was employed by Akinyoola et al.[40] and lower value of specific activity (0.25 HU/mg) and purification fold (3.1) were obtained. In another publication by Podder et al.,[29] a single purification step was used for purification of *Manikara zapota* lectin. The lectin was purified on affinity chromatographic column and higher value of specific activity (420 HU/mg) and purification fold (17.5) were reported. Chan and Ng[43] engaged anion exchange chromatography and gel filtration for the purification of *D. opposite* tuber lectin. The specific activity was higher than reported value in the current study.

DPH was found to resist heat denaturation up to 60 °C, as it was found to maintain full hemagglutinating activity up to 60 °C even after heating for one hour. Although, at 70 °C, the hemagglutinin lost 50% of its hemagglutinating activity after 1 hour of incubation but was still found to maintain its native hemagglutinating activity after heating for 30 minutesat this temperature and up to 90 °C before totally losing it at 100 °C

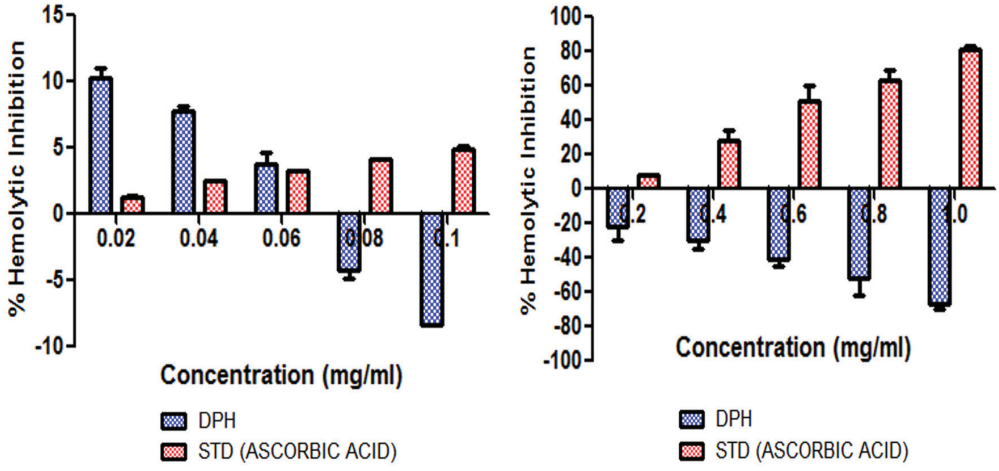

Figure 4. Anti-hemolytic potential of DPH
DPH: *Dioscorea preussii* hemagglutinin

(Figure 2A). Lectins isolated from *D. opposita by* Chan and Ng,[43] *Arisaema helleborifolium* Scott. by Kaur et al.,[44] and *Nymphaea nouchali* var. caerulea by Kabir et al.[45] were all shown to resist thermal inactivation till up to 60 °C. The loss of hemagglutinating activity of *D. preussii* at high temperatures might be since the weak interactions responsible for maintaining the structural integrity of hemagglutinin were disrupted, thereby altering its native conformation responsible for its activity.[46,47]

The pH is a consequence of the amino acid composition of the protein and is noticed in almost all enzyme reactions and in some proteins' functions. Hydrogen or hydroxyl ions are highly reactive and can interact with the most ionizable groups present at the surface of the protein molecule, possibly at the active center. Therefore, any pH alteration is connected to a change in the ionization state of the molecule, which in turn regulates the binding forces between the enzyme and substrate.[48] DPH was found to show high tolerance to a wide pH range. Although it had 50% hemagglutinating activity at pH 3 and 4, it was found to maintain full hemagglutinating activity at pH 5-13 (Figure 2B). It can be speculated that an increase in hydroxyl ions favored lectin better ionization, thereby promoting good binding forces between hemagglutinin and the erythrocyte membrane, which eventually led to the stability of hemagglutinating activity. This is close to what was reported for *D. opposita* tuber lectin, whose stability was manifested over a wide range of pH (pH 2-13), but lost 50% of its activity at pH 0-1.[43] Kaur et al.[38] also documented that the tuber lectin of *C. bicolor* exhibits hemagglutinating activity in a wide pH range of 2.5-12.5. DPH has a wider pH range stability than *D. mangenotiana* tuber lectin, which was reported by Akinyoola et al.[40] *D. mangenotiana* lectin activity was stable between pH 3 and 8, and lost approximately 12.5% of its activity at pH 9 and above.

While some lectins are known to require the presence of one or more metal ions, especially divalent cations, to exhibit their full hemagglutinating activity,[29,49,50] others do not require them because they still possess their optimal activities even in the absence of these metals. Using chelating agents like EDTA may lead to a decrease in or complete loss of hemagglutinating activity if the lectin requires a metal ion for its activity. In the chelating study conducted in this research, it was discovered that the hemagglutinating activity of DPH was not affected by the treatment of the metal chelating agent EDTA (10 mM and 50 mM). It might be an indication that DPH does not require the presence of metal ions in order to carry out its hemagglutinating activity or that these metal ions are too tightly bound to the hemagglutinin so much that EDTA could not remove them. This is comparable to the observations of Dhuna et al.,[37] Akinyoola et al.[40] and Pereira et al.[42] on tuber lectins from *A. utile, D. mangenotiana,* and *C. esculenta.* The authors documented the inability of EDTA to affect the hemagglutinating activity of lectins extracted from the tubers. Contrary to the above reports, Kabir et al.[45] reveals that the hemagglutinating activity of *N. nouchali* tuber lectin requires the presence of Ca^{2+}, Ba^{2+}, or Mg^{2+}.

A protein's tertiary structure is maintained by both covalent and non-covalent interactions, which include hydrogen bonds, hydrophobic bonds, ionic interactions, van der Waal's forces, and disulfide linkages. Introducing certain denaturing agents, such as a disulfide bond breaking agent (β-Mercaptoethanol) and chaotropic agents (guanidine-HCl and Urea) might give an insight into some structural properties of the protein. All denaturing agents used in this study reduced the hemagglutinating activity of hemagglutinin to different degrees, as shown in Figure 2C. The decrease in activity observed with the addition of β-Mercaptoethanol, might be an indication that the hemagglutinin contains a disulfide bond crucial to its hemagglutinating activity, which β-Mercaptoethanol reduces. The reduction in the hemagglutinating activity of hemagglutinin due to chaotropic agents (guanidine-HCl and urea) might indicate that hemagglutinin is a globular protein whose hydrogen bonds and hydrophobic interactions are disrupted by these agents.[51] The findings of the present study are consistent with those of other researchers on tuber lectins.[37,38,40,45,47]

During metabolic reactions, different free radicals are generated, but the most significant are those produced from oxygen, which are designated as ROS. Major biomolecules (DNA, lipids and proteins) can have their integrity damaged

by excess ROS, thereby causing an increase in the oxidative stress found in different human disease conditions.[14] In order to prevent these destructive effects of free radicals, the human body uses antioxidants, which can be endogenous (produced within the body) or exogenous (supplied to the body from foods and supplements), to neutralize them through different mechanisms, including donating electron(s) to reactive species, chelating metals, hydrogen donation, enzyme inhibition, and peroxide decomposition.[15,16]

The DPPH radical scavenging assay is one of the most widely used antioxidant assays for determining the antioxidant activity of natural samples/compounds. 2,2-diphenyl-1-picrylhydrazyl, which is the radical form of DPPH, accepts a proton from a donor to become the stable form 2,2-diphenyl-1-picrylhydrazine, which is accompanied by a change of color from deep violet to yellow.[52] This assay takes advantage of this color change that occurred only after the reduction of DPPH to monitor spectrophotometrically at 517 nm the radical scavenging capacity of a sample.[52] DPH was evaluated to have a dose-dependent free radical-scavenging activity (Figure 3A) with an IC_{50} of 0.727 ± 0.035 mg/mL, which was lower in activity than that of ascorbic acid, which had an IC_{50} of 0.022 ± 0.001 mg/mL.

A reducing power assay is typically used to assess the ability of a substance to donate an electron. DPH was observed to reduce Fe^{3+}/ferriccyanide complex to the Fe^{2+}/ferrouscyanide form, as seen through the change in color to blue measured at 593 nm. The FRAP assay showed that hemagglutinin contained 76 mg of AAE per gram of partially purified hemagglutinin.

The ability of DPH to chelate ferrous (a transition metallic ion) was determined using a metal chelating activity assay. Ferrous, like Cu^{+}, is a prooxidant known to be capable of generating hydroxyl radicals, very destructive ROS, from hydrogen peroxide through a reaction known as the Fenton reaction. A ferrozine-Fe^{2+} complex is formed when Fe^{2+} and ferrozine react together.[53] The addition of hemagglutinin disrupted complex formation, reducing the strength of the color that was measured at 562 nm. The reduction in color intensity was an indication that the hemagglutinin was able to chelate ferrous ions, thereby showing the potential to prevent the formation of dangerous hydroxyl free radicals. DPH was able to chelate iron in a dose-dependent manner (Figure 3B) with an IC_{50} of 0.583 ± 0.078 mg/mL, whereas the standard (EDTA) had an IC_{50} of 0.041 ± 0.006 mg/mL.

The ability of hemagglutinin to prevent lipid peroxidation was also investigated as a potential antioxidant. When lipid peroxidation, which is known to disrupt the cell membrane and lead to cell damage, occurs in the biological system, aldehydes such as malondialdehyde (MDA) are produced.[54,55] MDA is known to be very reactive, and it is typically used as an indicator of tissue damage. The colorless MDA can react with thiobarbituric acid to produce a pink adduct that can be measured at 532 nm spectrophotometrically. In the presence of an antioxidant, oxidation is inhibited, resulting in a reduction in absorbance. It was observed from the result of this assay that DPH, unlike the standard (BHT), was not found to possess anti-lipid peroxidation activity. Hemagglutinin could not inhibit lipid peroxidation. Lipid peroxidation levels increased as the amount of hemagglutinin increased from 0.2 to 1 mg/mL (Figure 3C). Unlike BHT, it prevents lipid peroxidation in a dose-related manner. The concentration of BHT increased with increasing percentage inhibition of lipid peroxidation.

Erythrocytes are known to have membranes containing abundant unsaturated fatty acids. The concentrations of oxygen they contain are very high; therefore, they are exposed to oxygen more than any other tissue in the body, making them more vulnerable to oxidative injury. They are therefore universal candidates for studying membrane hemolysis due to free radical attack. Exposure of erythrocytes to oxidative stress can result in protein damage, lipid peroxidation, and eventually hemolysis. Hydrogen peroxide is a well-known ROS that is highly stable and involved in signaling cascades and diffusion, making it to be considered an attractive oxidant model.[18,19,56] The antihemolytic assay gave biphasic results. At lower concentrations, hemagglutinin inhibited cell membrane hemolysis in the presence of an oxidative stress agent, as the concentration increased, this ability was aborted. The amount of hemoglobin released when H_2O_2 was introduced continued to increase as the hemagglutinin concentration increased (Figure 4). Although in the simple hemolytic assay conducted in the absence of a hemolytic agent, hemagglutinin provided a reasonable level of cell membrane protection with 4.2%-7.0% hemoglobin released in the concentration range of 0.2-1.0 mg/mL.

CONCLUSION

In conclusion, this study reported a novel hemagglutinin from the root tuber of *D. preussii*, which is a starch-binding lectin that showed physicochemical properties similar to those of other *Dioscorea* species lectins and also possessed antioxidant and cell membrane protective activities, which can be of great health benefit.

Ethics

Ethics Committee Approval: The study approved by the Obafemi Awolowo University Departmental Health Research Ethic Committee (approval number: OAUBCH/HREC/2024/005, date: l4.08.2024).

Informed Consent: Not required.

Authorship Contributions

Concept: O.O.O., Design: O.O.O., S.I.A., Data Collection or Processing: S.I.A., Analysis or Interpretation: O.O.O., S.I.A., Literature Search: O.O.O., S.I.A., Writing: O.O.O.

Conflict of Interest: No conflict of interest was declared by the authors.

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