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Hemagglutinin from the Root Tuber of *Dioscorea preussii* Pax Exhibit Antioxidative Prowess and Haemolysis Inhibition

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Abstract

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

Material and Methods: Hemagglutinating assay was used to detect the presence of lectin in the Phosphate Buffer Saline (PBS) extract of the *D. preussii* root tuber. The lectin was purified using ammonium sulphate fractionation and molecular sieve chromatography. Optimum pH and temperature were determined. So also, antioxidant activity was assessed using DPPH radical scavenging, metal chelating, FRAP and lipid peroxidation inhibition assays. Red blood cells subjected to oxidative damage caused by H_2O_2 was employed to evaluate its antihemolytic ability.

Results: Starch inhibited the haemagglutinating activity of the hemagglutinin. *Dioscorea preussii* haemagglutinin (DPH) maintained full haemagglutinating activity from 30 ºC to 60 ºC and pH 5-13. EDTA had no effect on the haemagglutinating activity of the haemagglutinin. All the denaturing agents (Guanidine-HCl, Urea and β-Mercaptoethanol) reduced the haemagglutinating activity of the haemagglutinin to different degrees. The haemagglutinin scavenged DPPH radical and chelated iron metal with IC_{50} of 0.727 ± 0.035 mg/ml and 0.583 ± 0.078 mg/ml respectively, while the FRAP assay showed that it contains 76 mg of ascorbic acid

equivalent per g of the purified haemagglutinin. In absence of hemolytic agent, minimal haemolysis was recorded but the hemagglutinin could not protect the cell membrane in the presence oxidative agents. Although, at lower concentration tested, the hemagglutinin shows positivity in membrane integrity protection.
Conclusion: The 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 make it a novel lectin.
Keywords: *Dioscorea preussii,* hemagglutinin, antioxidant, anti-haemolytic, lectin

INTRODUCTION

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

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

Very little is known about *Dioscorea preussii* Pax as extremely few studies have been done on this wild plant. It is a climber whose stem twines left-handedly, with the ability of going up to 24 m, from its narrowly 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. The tubers are mostly eaten in some places during famine.[5,6] In Nigeria, the tuber is known as ainyelo, igioruesi and esuru-igbo among the Igbo, Edo and Yoruba people respectively.[5,7] The extract of *D. preussii* has been reported to show *in-vitro* cytotoxicity, antileishmanial and antifungal activities. Tabopda et al.[8] reported isolation and characterization of three important steroidal saponins from this tuber.

During cellular metabolism, which is a normal cellular activity, free radicals are produced. These free radicals can simply be described as atoms or molecules containing lone electron or more in their outer orbits, making atoms or molecules to be unstable and highly reactive. These reactive species at low or moderate levels perform beneficial roles such as in immunity, redox regulation, cellular signaling pathways and in mitogenic response.[9] At higher concentrations, reactive oxygen species (ROS) bring about oxidative stress while reactive nitrogen species (RNS) cause nitrosative stress, which are capable of destroying biomolecules. Biomolecules such as 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.[9]

To prevent destructive effects of free radicals, the human body has devised different mechanisms to combat them through certain agents known as antioxidants.[10] Antioxidants are molecules that

can neutralize free radicals in order to prevent them from causing cellular damage. They can do this by donating electron(s) to reactive species, chelating metals, hydrogen donation, enzyme inhibition and peroxide decomposition.[10,11]

Haemolysis is the rupturing of the erythrocytes.[12] The red blood cells are known to possess abundant polyunsaturated fatty acids, a high concentration of oxygen, haemoglobin and membrane proteins. Because erythrocytes are more exposed to oxygen more than any other cell or tissue in the body, they are more vulnerable to oxidative destruction, so they are frequently and widely used in the study of oxidative damage in membranes. Moreover, while haemoglobins present in erythrocytes are strong catalysts that can lead to the initiation of lipid peroxidation, the invasion of the membranes of erythrocytes by peroxidants can also result in cell haemolysis.[13,14] This is because, when the peroxidation of the membrane unsaturated fatty acids occurs, this leads to the disruption of the usual organization of the membrane lipids, resulting in membrane pore formation and alteration in water permeability, eventually damaging the membrane structure leading to haemolysis.[15] Because of the relatively high stability and diffusion of hydrogen peroxide, it is considered to be an awesome oxidant model used in the investigation of both the haemolytic and anti-haemolytic activities of various samples.[14] Different natural substances have been proposed to be used as therapeutic agents in order to prevent haemolysis, some of whose anti-haemolytic potential has been linked to their free radical scavenging activity.[16,17]

Lectins are ubiquitous (glyco)proteins that possess at least one binding site for carbohydrate or its derivate, have no catalytic function, and are also of non-immunoglobulin origin.[18] All these proteins were initially referred to as haemagglutinins, a term now solely for those whose sugar specificities are yet to be known or discovered. Plants are a great source of lectins, and it also represents the major source where lectins that are analyzed are being isolated from. Lectins are known to have so many biological importance or activities including antifungal, antitumor, antibacterial, antiviral, termicidal, and insecticidal activities.[19] Lectins are also known to perform various roles including storage, transporting, signaling,[20,21] cell recognition, cell migration, endocytosis, complement activation, cell adhesion, intracellular translocation processes, apoptosis activation, cell signaling, immune regulation and defense against pathogens.[20,22]

Under the condition of severe redox imbalance, erythrocytes become vulnerable and cellular defense does not offer full defense from the attack of reactive and free radicals, which could lead to oxidative damage related-diseases such as cardiovascular diseases. Recently haemagglutinin, which possesses antioxidant activity, has been reported from plant sources and also protects erythrocytes from haemolysis. The literature search indicates that there is still a scarcity of plant-derived proteins with such activity. Therefore, identifying haemagglutinin with antioxidant potential and protective effect on erythrocytes, having little or no side effects will be of great benefits. The study led to purification of a novel haemagglutinin from root tuber of *D. preussii*, and the determination of the physicochemical properties of the haemagglutinin. It also investigated the antioxidant and anti-haemolytic activities of the haemagglutinin, with a view to exploring the therapeutic potential of the haemagglutinin.

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 from rabbit were also obtained from

apparently healthy rabbit bought from Teaching and Research Farm, Obafemi Awolowo University, Ile Ife, Nigeria.

Preparation of Dioscorea preussii crude extracts

The root tubers of *D. preussii* were collected from a farm within Obafemi Awolowo University, Ile-Ife, Nigeria. The root tubers of *D. preussii* were thoroughly washed to remove some sand particles and peeled. The peeled tubers were minced into very small pieces, and then ground into a fine paste. The ground tubers were extracted at 4° C overnight with 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 was herein and after termed crude extract.

Glutaraldehyde fixation of erythrocytes

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 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 the fixation, the mixture was centrifuged for 5 minutes 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.

Haemagglutinating assay

The presence of lectin in the crude extract of *D. preussii* and various fractions was determined through a modified haemagglutinating assay procedure of Odekanyin and Kuku.[23] 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 any of the 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 microliters of the fixed erythrocyte suspension (50 µl) was pipetted into each well. The plate was left undisturbed for two hours on the laboratory bench and then observed for any visible haemagglutination. The control experiment was without extract or any of the fractions. Haemagglutination titre 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 titre units number per mg protein expressed as haemagglutinating units (HU)/mg.

Sugar specificity test

Sugar specificity of *D. preussii* haemagglutinin (DPH) was determined by comparing the abilities of different sugars to inhibit the haemagglutinating activity of the haemagglutinin. Firstly, the serial dilution of the haemagglutinin was carried out as described in section 2.5 until the last dilution where haemagglutination 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 hours. 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 haemagglutination. 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[24] of protein concentration determination was adopted to determine the total protein concentration of the crude extract and other fractions. Bovine serum albumin (BSA) was employed as standard.

Purification of lectin

Ammonium sulphate fractionation

Solid ammonium sulphate (16.4 g/100 ml) was mixed to a known volume of the crude extract with gentle stirring, to bring the solution to 30 % ammonium sulphate 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 sulphate fraction. The ammonium sulphate saturation of the supernatant was then increased to 60 % through the slow addition with gentle stirring of solid ammonium sulphate (18.1 g/100 ml). The precipitate was also collected and represented 60 % ammonium sulphate precipitate fraction. Again, the ammonium sulphate saturation of the supernatant was raised to 90 % by slowly adding solid ammonium sulphate (20.1 g/100 ml). The precipitate representing the 90 % fraction was likewise collected. All precipitates were separately dialyzed against PBS exhaustively and the dialysate of each fraction was tested for haemagglutinating activity. The dialysates were later stored in the deep freezer (below -4 ºC)

Gel-filtration on Sephadex G-100

About 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 the *D. preussii* ammonium sulphate dialysate was layered on the packed column, the same buffer (PBS) was used in eluting proteins, while collecting 5 ml fractions at 20 ml/hr flow rate. The haemagglutinating activity and protein concentration of these fractions were evaluated. Fractions with high haemagglutinating 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 the lectin

Temperature effect on haemagglutinating activity

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

pH effect on haemagglutinating activity

Aliquot of the purified haemagglutinin was incubated for an hour with various buffers having different pH values to evaluate the effect of pH on the haemagglutinating activity of purified haemagglutinin. The same concentration (0.2 M) of the different buffers was used, and the buffers used and their pH range are 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 haemagglutinin were subjected to hemagglutinating assay after the incubation to determine the residual activity.

Denaturants effect on haemagglutinating activity

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

Chelating agents' effect on haemagglutinating activity

Purified haemagglutinin was dialyzed against two different concentrations of EDTA (10 mM and 50 mM) separately for about 24 hours at 4 °C to determine the effect of EDTA (Ethylene diamine tetraacetic acid) on DPH. After which it was assayed for haemagglutinating activity.

Antioxidants assays

Scavenging of DPPH radical assay

The purified haemagglutinin was assessed for its ability to scavenge DPPH radical according to the method of Huh and Han.[25] Three tenths millimolar DPPH in methanol (1 ml) was mixed with varying concentrations of the haemagglutinin (sample)/Ascorbic acid (standard), after which the mixture was incubated for half an hour in the dark cupboard. Absorbance reading was taken at 517 nm using Biobase UV-visible spectrophotometer BK-D5 series against control in which sample/standard was substituted with methanol.

The formula below was used in calculating percentage inhibition:

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

While $A_{control}$ 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,[26] 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 $FeCl_3.6H_2O$ in the ratio 10:1:1 respectively, were mixed together. Varying concentration of ascorbic acid (50 µl, 0.02-0.1 mg/ml) or haemagglutinin (50 µl of 0.1 mg/ml) was added to FRAP (1 ml). Ten minutes after mixing, absorbance was measured at 593 nm against the reagent blank. The samples were protected from direct sunlight. The ascorbic acid equivalent (AAE) of the sample was estimated. The equivalent concentration (EC) was used in expressing the reducing power.

Lipid peroxidation inhibition assay

This assay was performed in accordance with the described procedure of Masao *et al.*[27] 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 ºC for an hour. Thereafter, 0.67 % thiobarbituric acid (TBA, 2.0 ml) was added. The mixtures were incubated for 30 mins at 100 ºC, allowed to cool, with the subsequent addition of butan-1-ol (2.0 ml), and centrifuged for 10 mins at 3000 rpm. The absorbance of 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:

$$\text{Lipid Peroxidation Inhibition} = \frac{A - B}{A} \times 100$$

While 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 *D. preussii* haemagglutinin to form a complex with iron was determined using metal chelating assay. The method of Singh and Rajini[28] was used with minor modifications. Stock solutions of 2 mM $FeSO_4 \cdot 7H_2O$ and 5 mM ferrozine were diluted 20 times. $FeSO_4 \cdot 7H_2O$ (1 ml) was combined with varied concentrations of the haemagglutinin (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. Thereafter, absorbance of the solution was taken at 562 nm spectrophotometrically. The formula below was used in calculating the percentage inhibition of the formation of ferrozine-Fe^{2+}:

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

$A_{control}$ = the absorbance of the control,
A_{sample} = the absorbance of the tested samples.

Anti-haemolytic activity assay

The human blood group O-positive used in carrying out this assay was first prepared before being used. The blood drawn from a healthy donor was washed five times with PBS using centrifugation. Five percent (5 %) of the washed erythrocytes were then prepared in PBS. Ebrahimzadeh *et al*.[29] procedure was adopted with little modification to determine the anti-haemolytic activity. Fifty microlitres of different concentrations of haemagglutinin/standard (Ascorbic acid) were added to the erythrocytes suspension in PBS (5 %, 100 µl). After 30 mins 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 gently shaken of the mixture. After the incubation, the reaction mixture was centrifuged for 10 mins at 2500 rpm. The supernatant was collected and then the absorbance was read at 540 nm, to estimate the amount of hemoglobin released. The erythrocytes were also treated as above, but without the haemagglutinin to obtain complete haemolysis taken as 100 %.

In evaluating the hemolysis induced by the sample (hemagglutinin), red blood cells were pre-incubated with the sample (hemagglutinin, 50ul), after which amount of hemoglobin released was determined. While the percentage of hemolysis was calculated by using the formula:

The formula below was used in calculating the percentage anti-haemolysis:

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

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

Haemolysis due to H_2O_2 (100 µl) was taken as 100 % haemolysis
A_1 represents absorbance of tested samples
A_2 represents absorbance of 100 % haemolysis

Statistical analysis

The experiments were done in triplicates and the results were expressed as Mean ± Standard error of mean (SEM) and analysed using One-way analysis of variance (ANOVA) for multiple measurement. Graphpad Prism statistical software version 7.0 (San Diego, California, 92108, United States) was used for all the statistical analysis in this study.

RESULTS AND DISCUSSION

In this study, the crude extract obtained from the root tubers of *D. preussii* was observed to contain hemagglutinin. The hemagglutinin was isolated and purified and termed *D. preussii* hemagglutinin (DPH). *Dioscorea preussii* hemagglutinin hemagglutinating activity was non-specific against all the types of red blood cells used, as it agglutinated all the human blood group (ABO) and rabbit erythrocytes as shown in **Table 1**. This result showed that the lectin possessed blood group specificity different from the majority of tuber lectins. The tuber lectins are majorly rabbit erythrocytes specific. Tuber lectins from *Arisaema utile* Hook.f. Scott,[30] *Caladium bicolor* (Aiton) Vent.,[31] and Dioscorea *bulbifera* L.[32] have all been reported to agglutinate only rabbit erythrocytes but unreactive to human ABO blood group erythrocytes. Recently, contrary report to this was published by Akinyoola et al.[33] Their report showed that the tuber lectin of *Dioscorea mangenotiana* J.Miège agglutinated all the human blood group (ABO) and rabbit erythrocytes non-specifically. This is consistent with the results reported in this current study. Also in support of this, Sharma et al.[34] published isolation and purification of *Adenia hondala* (Gaertn.) de Wilde tuber lectin, a human blood group non-specific that agglutinated rabbit erythrocytes.

Hapten inhibition assay, which determine the sugars ability to inhibit the hemagglutinating activity of the *D. preussii* tuber hemagglutinin, was carried out. Different sugars including monosaccharides, disaccharides, and their derivatives along with some polysaccharides were used for this study, but none of the sugars was found to inhibit the hemagglutinating activity of the hemagglutinin except starch. This is compatible with what Sharma et al.[32] reported that all the tested simple sugars derivatives and the simple sugar themselves did not inhibit the hemagglutinating activity of the aerial tuber of *D. bulbifera* lectin. Pereira et al.[35] also reported that all the tested simple sugars ~~could not inhibit~~ the hemagglutinating activity of the *Colocasia esculenta* lectin. Contrary to this was the report of Akinyoola et al.[33] where they revealed that glucose and N-acetylglucosamine were able to inhibit *D. mangenotiana* tuber lectin activity. Galactose was also found to inhibit *Dioscorea opposite* Thunb. tuber lectin activity.[36] It was discovered, in the current study, that polysaccharide of plant origin (starch) inhibited the hemagglutinating activity of the *D. preussii* tuber hemagglutinin.

The crude lectin extract from the root tubers of *D. preussii* was subjected to salt fractionation using ammonium sulphate. The dialysate of the 60 % ammonium sulphate precipitate was found to have higher hemagglutinating activity than the dialysates of the 30 % and 90 % ammonium sulphate precipitates (Table 2), and therefore was purified and used for further studies. The sixty percent (60 %) ammonium sulphate precipitate dialysate gave one major protein peak with 2 minor peaks when it was gel-filtered on Sephadex G-100 column. The haemagglutinating activity was found to reside in the major protein peak. The fractions of the activity peak were pooled. The specific activity and fold purification 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. 30 % and 90 % ammonium sulphate precipitate dialysates were also gel-filtered. The active fractions were pooled and had insignificant haemagglutinating activity (Figure 1B and 1C).

Dioscorea preussii hemagglutinin was found to resist heat denaturation up to 60 ºC, as it was found to maintain full hemagglutinating activity up to 60 ºC even after being heated for an hour. Although, at 70 ºC, 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 minutes at this temperature and up to 90 ºC before totally losing it at 100 ºC (Figure 2A). Lectins isolated from *D. opposita by* Chan and Ng,[36] *Arisaema helleborifolium* Scohtt. by Kaur et al.,[37] and *Nymphaea nouchali* var. caerulea by Kabir et al.[38] were all shown to have resisted thermal inactivation till up to 60ºC. *Dioscorea preussii* hemagglutinin loss of hemagglutinating activity at high temperature, might be due to the fact that the weak interactions that were responsible for maintaining the hemagglutinin structural integrity were disrupted, thereby altering its native conformation responsible for its activity.[39,40]

Dependence of pH is a consequence of amino acid composition of the protein and is noticed in almost all enzyme reactions and some protein's functions. Hydrogen or hydroxyl ions are highly reactive and capable of interacting with the most ionizable groups present at the surface of the protein molecule and possibly at the active center. Therefore, any alteration in pH is connected with a change in the ionization state of the molecule which in turn, regulates the binding forces between the enzyme and substrate.[41] *Dioscorea preussii* hemagglutinin was found to show high tolerance to a wide pH range, while it had 50% hemagglutinating activity at pH 3 and 4, it was discovered to maintain full hemagglutinating activity at pH 5-13 (Figure 2B). It can be speculated an increase in hydroxyl ion favored the lectin better ionization, thereby promoting good binding forces between the hemagglutinin and erythrocyte membrane that 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.[36] Kaur et al.[31] also documented the tuber lectin of *C. bicolor* to exhibit hemagglutinating activity at a wide pH range of 2.5-12.5. But *D. preussii* hemagglutinin has wider pH range stability than *D. mangenotiana* tuber lectin which was reported by Akinyoola et al.[33] to have pH stability between pH 3-8, and then later losing its activity to about 12.5% at pH 9 and above.

While some lectins are known to require the presence of one or more metal ions especially the divalent cations, to exhibit their full hemagglutinating activity, others do not require them as they still possess their optimal activities even in the absence of these metals. Using chelating agents like EDTA may lead to decrease in or complete loss of hemagglutinating activity if the lectin requires metal ion for its activity. In the chelating study carried out in this research, it was discovered that the hemagglutinating activity of *D. preussii* hemagglutinin was not in any way affected after dialyzing it against the metal chelating agent, EDTA (10 mM and 50 mM). It might be an indication that *D. preussii* hemagglutinin 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 the EDTA could not remove them. This is comparable to the observations made by Dhuna et al.,[30] Akinyoola et al.[33] and Pereira et al.[35] on tuber lectins from *A. utile, D. mangenotiana* and *C. esculenta,* respectively. They documented the inability of EDTA to affect the hemagglutinating activity of lectins extracted from the tubers. Contrary to the

above reports, Kabir et al.[38] revealed that the hemagglutinating activity of *N. nouchali* tuber lectin required 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 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 the denaturing agents used in this study reduced the hemagglutinating activity of the hemagglutinin to different degrees as shown in Figure 2C. The decrease in activity observed on account of 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. While the reduction in the hemagglutinating activity of the hemagglutinin due to the chaotropic agents (Guanidine-HCl and Urea) might infer that the hemagglutinin is a globular protein whose hydrogen bonds and hydrophobic interactions were disrupted by the chaotropic agents.[42] The findings of the present study are consistent with the results of other researchers on some tuber lectins.[30,31,33,38,40]

During metabolic reactions, different free radicals are generated, but the most significant are those produced from oxygen, which are designated as reactive oxygen species. Major biomolecules (DNA, lipids and proteins) could have their integrity damaged by the excess reactive oxygen species, thereby causing an increase in the oxidative stress found in different human disease conditions.[9] 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.[10,11]

DPPH radical scavenging assay is one of the most widely antioxidant assays used in 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, being accompanied with a change of colour from deep violet to yellow.[44] This assay takes advantage of this colour changed that occurred only after the DPPH has been reduced, to monitor spectrophotometrically at 517 nm the radical scavenging capacity of a sample.[44] *Dioscorea preussii* haemagglutinin was discovered to have free radical scavenging activity that was dose dependent (Figure 3A) with an IC_{50} of 0.727 ± 0.035 mg/ml which was lower in activity than that of the Ascorbic acid having an IC_{50} of 0.022 ± 0.001 mg/ml.

The reducing power assay is usually employed in assessing the ability of a substance to donate an electron. *Dioscorea preussii* haemagglutinin was observed to reduce Fe^{3+}/ferriccyanide complex to the Fe^{2+}/ferrouscyanide form as was seen through the change in colour to blue that was measured at 593 nm. The FRAP assay showed that the haemagglutinin contained 76 mg of ascorbic acid equivalent per gram of the partially purified haemagglutinin.

The ability of *D. preussii* haemagglutinin to chelate ferrous (a transition metallic ion) was determined through metal chelating activity assay. Ferrous like Cu^{+}, is a prooxidant known to be capable of generating hydroxyl radical, very destructive ROS, from hydrogen peroxide through a reaction known as Fenton reaction. A ferrozine-Fe^{2+} complex is formed when Fe^{2+} and ferrozine react together.[45] The addition of the hemagglutinin disrupted the complex formation which reduced the strength of the colour that was measured at 562 nm. The colour intensity reduction was an indication that the haemagglutinin was able to chelate ferrous ion, thereby showing the potential of being able to prevent the formation of the very dangerous hydroxyl free radical.

Dioscorea preussii haemagglutinin was able to chelate iron in a dose dependent manner (Figure 3B) with an IC_{50} of 0.583 ± 0.078 mg/ml, while the standard (EDTA) had an IC_{50} of 0.041 ± 0.006 mg/ml.

The ability of the hemagglutinin to prevent lipid peroxidation was also used to investigate its antioxidant potential. When lipid peroxidation which is known to disrupt the cell membrane and also lead to cell damage occurs in the biological system, aldehydes such as malondialdehyde (MDA) are produced.[46,47] This MDA is known to be very reactive and it is usually used as an indicator to detect tissue damage. The colourless 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, the oxidation is inhibited, that will result in reduction of the absorbance. It was observed from the result of this assay that *D. preussii* hemagglutinin unlike the standard (Butylated hydroxyltoluene, BHT) was not found to possess anti-lipid peroxidation activity. The hemagglutinin was unable to inhibit the lipid peroxidation. The lipid peroxidation level was found to rise as the amount of the hemagglutinin increased from 0.2 mg/ml to 1 mg/ml (Figure 3C). Unlike BHT, that prevents lipid peroxidation in a dose related mode. The concentration of BHT increased with percentage inhibition of lipid peroxidation.

Erythrocytes are known to have membranes with abundant unsaturated fatty acids. The concentrations of oxygen they contain is very high and are therefore more exposed to oxygen more than any other tissue in the body, making them more vulnerable to oxidative injury. They are therefore universal candidate in studying membrane hemolysis due to free radical attacks. Exposure of erythrocytes to oxidative stress can result in protein damage, lipid peroxidation, and eventually hemolysis. Hydrogen peroxide is well-known ROS that is highly stable, involved in signaling cascades, and diffusion, making it to be considered a very attractive oxidant model.[13,14,48] The antihemolytic assay gave biphasic results. At lower concentration, the hemagglutinin was able to inhibit cell membrane haemolysis in the presence of oxidative stress agent, as the concentration increased the ability was aborted. The amount of hemoglobin released when H_2O_2 was introduced keep increasing as the hemagglutinin concentration rises (Figure 4A and 4B). Although, in simple hemolytic assay conducted, in the absence of hemolytic agent, the hemagglutinin provided a reasonable level of cell membrane protection with 4.2% - 7.0% hemoglobin released at the concentration range of 0.2 mg/ml – 1.0 mg/ml.

CONCLUSION

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

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Table 1: Blood Group Specificity of *Dioscorea preussii* Crude Extract

Blood Type (Erythrocyte)		Hemagglutinating titre
Human Blood group	A	2^{11}

	B	2^{11}
	O	2^{11}
	AB	2^{11}
Rabbit		2^{11}

Table 2: Hemagglutinating Activities of Dialysates of 30%, 60% and 90% Ammonium Sulphate Precipitates of *Dioscorea preussii*

Dialysates	Hemagglutinating Titre
30%	2^6
60%	10
90%	2^5

Table 3: Purification Table of haemagglutinin from *Dioscorea preussii* tuber

Fractions	Total Protein (mg)	Total Activity (HU)	Specific Activity (HU/mg)	% Yield	Purification Fold
Crude Extract	233.10	2048	8.79	100	1
Ammonium Sulphate 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

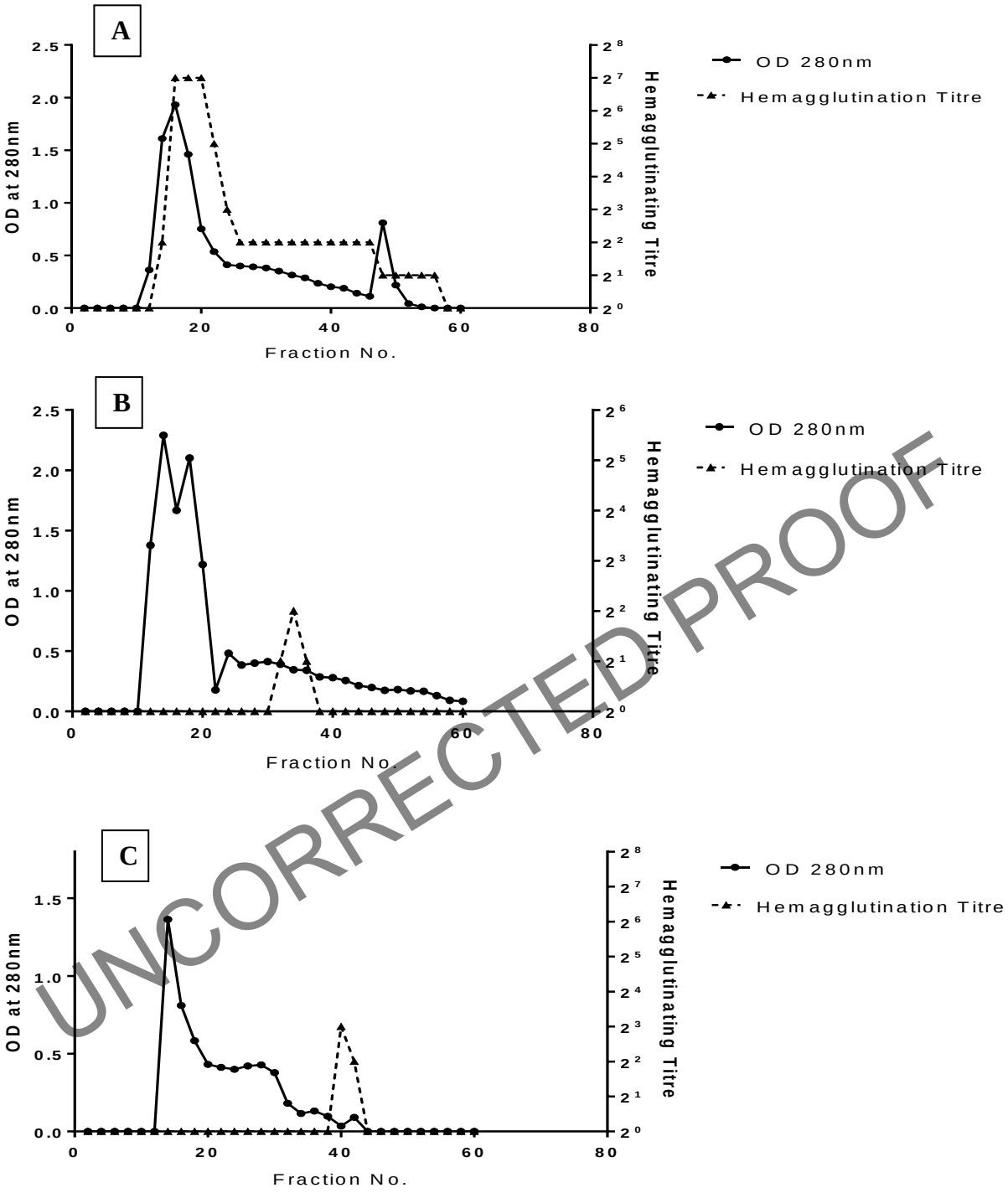

Figure 1: Gel Filtration Chromatogram of the Dialysate of the 60% [A], 30% [B], and 90% [C] $(NH_4)_2SO_4$ Precipitate of *D. preussii* crude extract on Sephadex G-100.

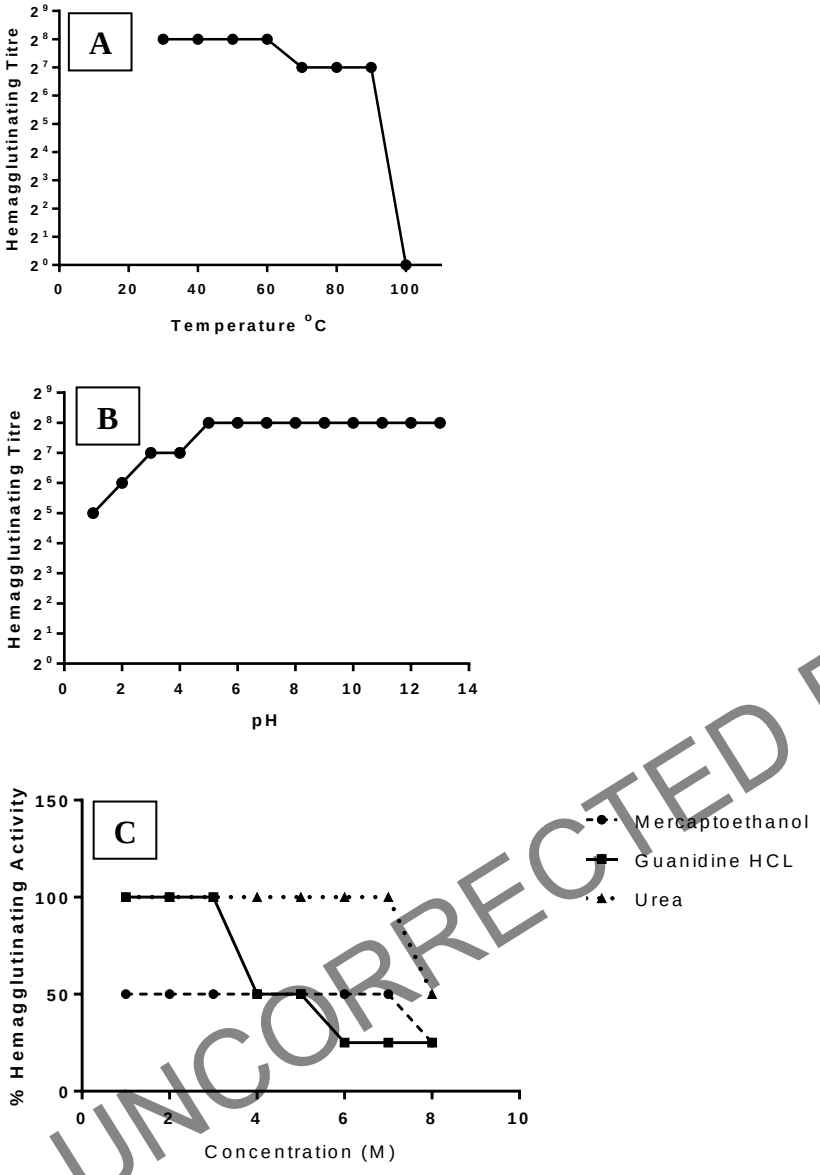

Figure 2: A - Effect of Temperature, **B -** Effect of pH and **C** - Effect of Denaturing Agents on DPH Hemagglutinating Activity.

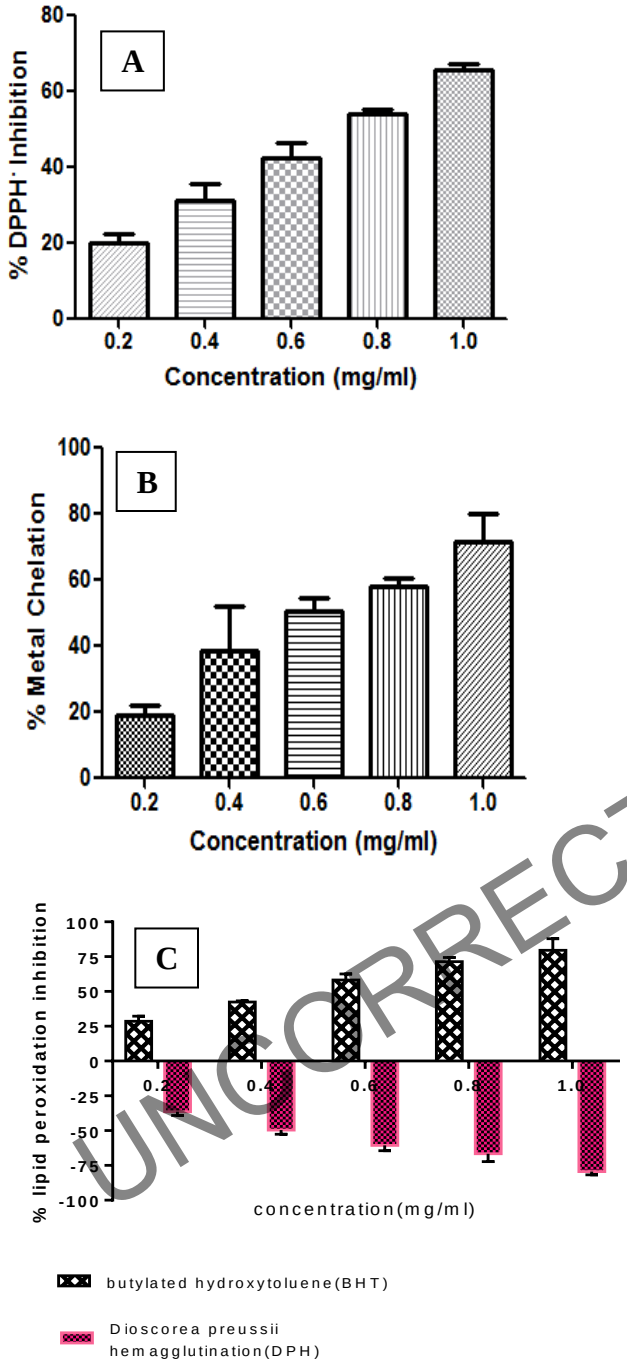

Figure 3: Antioxidant activity of *Dioscorea preussii* Hemagglutinin. **A -** DPPH Radical Scavenging Activity, **B -** Metal Chelating Activity, **C: -** Anti-lipid peroxidation Activity

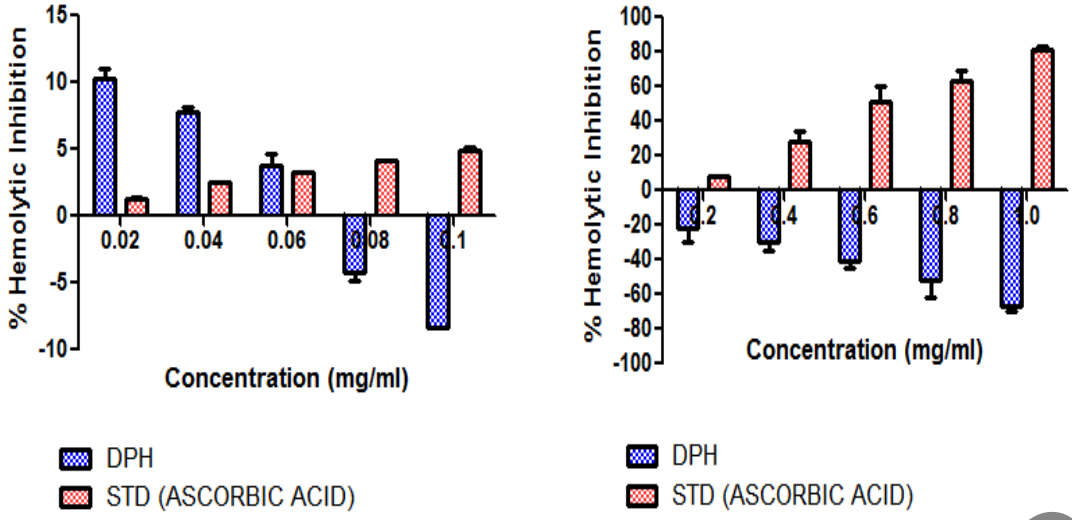

Figure 4A and B: Anti-hemolytic potential of *Dioscorea preussii* Hemagglutinin