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The Antisickling Effects of Extracts of Some Selected Vegetables from South-East Nigeria

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Abstract

The work investigated the effects of extracts of leaves of some selected vegetables consumed in South-East Nigeria for their antisickling potency. The vegetable samples included: Mucuna pruriens (Agbala), *Pterocarpus santalinoides* (Nturukpa), *Pterocarpus mildbreadi* (Oha) and *Piper methysticum* (Awa). The phytochemical compositions of the vegetables were quantified as well as the amino acid composition of the leaves extracts using standard methods. The samples were fractionated into crude aqueous extract (CAE), fat-soluble extract (FAS), butanol-soluble extract(BUS) and water- soluble extracts (WAS) respectively. Results of quantitative phytochemical yield show the following: For *P. mildbreadi*, flavonoid (4.05±0.0%), and terpenoids (3.01±0.0%); for *P. methysticum*, flavonoid(8.38±0.00%) and alkaloid (4.79±0.0%); *M. pruriens* was also rich in flavonoids (10.74±0.0%) and alkaloids (5.08±0.00%). The ascorbic acid concentrations of the leafy extracts are as follows: The mean value of Ascorbic acid is highest in *M. pruriens* (109.17±0.00) in mg/100g of sample and *P. santilinoides* (59.33±0.00)mg/100g. Nutritionally, the different leafy vegetable extracts were found to be rich sources of free amino acids and vitamin C. In haemoglobin polymerization inhibition experiment, the relative % inhibition of sickle cell haemoglobin polymerization by each extract was significant ($p<0.05$). For *P. mildbreadi* (12.99±0.00%); *P methysticum* (57.14±0.00 %), *M. pruriens*(25.97±0.00) and *P.santilinoides* (51.95±0.00). The Fe2+/ Fe3+ ratio analysis of the extracts on sickle cell blood revealed an increase for the crude aqueous extracts of the samples *: P santilinoides* (8.54%),*M. pruriens* (76.32%), *P . mildbreadi* (108.86%) and a decrease by the same aqueous extract of *P. methysticum* (-16.32%). Substantial concentrations of amino acids were found in the extracts of the vegetables, some of these include-Arginine, Histidine, Lysine, Methionine, Phenylalanine, Tryptophan, Tyrosine,Asparagine, Glutamine and others, most of which are antisickling amino acids.
Keywords: Sickle cell disease; Haemoglobin polymerization; Leafy vegetable extracts; Fe^{2+}/ Fe^{3+} ratio.

1. Introduction

Sickle cell disease is one of the most common genetic diseases world- wide and its highest prevalence occurs in the Middle East, Mediterranean regions, Southeast Asia and sub-Saharan Africa especially Nigeria. In many countries in Africa, 10-40 % of the population carry the sickle-cell gene resulting in an estimated SCD prevalence of 2 % [1].

Sickle cell disease is an increasing global health problem. Estimates suggest that every year, approximately 300,000 infants are born with sickle cell anemia, a genetic disorder affecting red blood cells (RBCs). At the genomic level, this is due to mutation in β-globin gene (HBB) resulting in the substitution of valine for glutamic acid at the sixth position of the globin gene resulting in hemoglobin S (HbS). Under hypoxic condition; HbS polymerizes, resulting in rigid and distorted red blood cells, termed ''sickle cells'' which cause impaired microcirculation, hemolysis and reduced life span. Numerous clinical manifestations of sickle cell disease include: pain, vaso-occlusive crisis, spleenic sequestration, acute chest syndrome, aplastic anemia, hemolytic anemia, priapism, hand and foot-syndrome, and stroke [2]. Several antisickling agents have been investigated and confirmed to possess ameliorative effect. For instance, Hydroxyurea (HU), has been shown to decrease the number and severity of sickle cell crisis by inducing fetal hemoglobin production in patients with sickle cell anemia (Cokie *et al*., 2005). Studies have shown that there were no specific therapy for this syndrome before 1970s. Subsequent studies have shown that patients with higher concentrations of fetal haemoglobin (HbF) in their red blood cells had less adverse clinical complications [3]. Many therapeutic substance have applied in the management of sickle cell disease such as Vaillin, pyridyl derivatives, acetyl-3,5-dibromosalicylic acid and 5-hydroxymethyl-2-furfural; these were not clinically accepted for the management of the disease [4, 5] . Despite its wide acceptance, hydroxyurea is moderately toxic especially when administered for a long time . Many other preparations have been in use in Nigeria for the management of this debilitating disease and these have gained wide acceptance, such as Ciklavit^{TM} discovered by the duo: Late Prof G.I. Ekeke, Department of Biochemistry, University of Port-Harcourt, Nigeria and Prof. R.N. Nwaoguikpe, Department of Biochemistry, Federal University of Technology, Owerri, Nigeria. The drug is now

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produced and marketed by Neimeth Pharmaceutical PLC, Nigeria. Another potential preparation is Nipprisan™, a combination of four medicinal plants exhibiting synergistic effect in inhibiting sickle cell crisis.

The role of medicinal plants in the treatment and management of diverse health problems is well documented. Among the commonly used plants in Nigeria and other African nations for the management of common ailments including sickle cell disease are the leaves of *Terminalia catapa* and *Carica papaya* [6]. Others include *Cajanus cajan* seeds , unripe fruits of *Carica papaya*, leaves of *Parquetina* nigrescens, leaves of *Citrus sinensis*, leaves of *Xanthoxyllum zanthoxyloides* [7], *Alchornea cordefolia* [8]; *Moringa oleifera* leaves [9] The increasing number of sickle cell patients globally especially in Nigeria and Africa and the high cost of pharmaceuticals prompted our interest and desire to continually search for remedy/remedies for the management of sickle cell disease since herbal remedies have provided potent pharmaceuticals for treatment of such diseases like malaria, measles and typhoid fever. Investigation of the antisickling properties of substances derived from indigenous plants is an attractive line of research. It is to this end that we analyze the ability of the extracts of some widely used ethnomedicinal plants such as: *Mucuna pruriens*(Agbala) , *Pterocarpus mildbreadi* (Oha), *Pterocarpus santalinoides*(Nturukpa) and *Piper methysticum* (Awa) to assess the potency of the extracts of samples to inhibit and reverse the polymerization of HbS blood.

Mucuna pruriens (Agbala) is a tropical legume, native to Africa and tropical Asia, widely naturalized and cultivated. The plant is notorious for the extreme itchiness it produces on contact especially, the dried pods. It has agricultural and horticultural values and used in herbalism. *Pterocarpus santalinoides* (Nturukpa) is a tree species in the legume family, *Fabaceae*. It has a remarkable bi-continental distribution, native to tropical West Africa and also to South America [10].The leaves are eaten as vegetable.

Pterocarpus mildbreadi (Oha) is a specie of *Pterocarpus* in the family of Fabaceae, native to centraland tropical West Africa. *Pterocarpus mildbreadi* is a common vegetable consumed mainly by the Ndi Igbo of Eastern Nigeria. It is used in the preparation of soups. Oha leaves contain minerals like magnesium, calcium, potassium, copper, iron, zinc, and manganese It is a source of vitamins A, B, C and a source of amino acids like lysine, glutamic acid and cysteine with low sodium content. It has antioxidant, hypocholesterolemic, chemo-protective and antibacterial properties. *Piper methysticum* is a crop of the Pacific Islands .It is consumed throughout the Pacific Ocean cultures of Polynesia for its sedating effect. It can be used for medical purposes to relieve depression and stress.

2. Materials and Methods

2.1. Quantitative Determination of Phytochemicals

This was carried out using the standard procedures of the analytical methods of the Association of Official Analytical Chemists (AOAC, 2004) for the determination of alkaloids, flavonoids, tannins , saponins, phenols, oxalates, phytate, glycoside and terpenoids respectively.

2.2. Determination of Ascorbic Acid Concentration (Vit.C) of the Extracts

The determination of Ascorbic acid concentration of the leafy extracts was carried out by the methods of Lambert and Muir, 1974

2.3. Determination of Total Free Amino Acid Concentrations of FAS, BUS, and WAS Fractions

The total concentration of the free amino acid concentration of the extracts was determined with 0.1% ninhydrin in acetone using Phenylalanine as standard 0.1% Ninhydrin in acetone in acetone was diluted with distilled water in the ratio 1:4. The water soluble extract was diluted 1:1 with water, the chloroform solution of (Oha) extract diluted 1:1 with methylated spirit and BUS extracts diluted 1:5 with ethanol. The process repeated for all samples. Exactly 20 µl each of the diluted extracts was added to 4 ml portions of the diluted Ninhydrin. The resultant solutions were heated to boiling for 5 min., cooled and the absorbance read in a Spectrophotometer at 540 nm using distilled water as blank. The values were obtained from a standard curve obtained by treating 20 µl portions of different concentrations (1-20 mg/ml) of Phenylalanine with 4 ml portions of diluted Ninhydrin and treated as above. A plot of absorbance against concentration in mg/ml and extrapolations made.

2.4. Preparation of Blood Sample for Hemoglobin Polymerization Experiment

The blood sample was collected from the Hematology Laboratory of the Federal Medical Centre, Owerri , Nigeria . 0.2 ml of whole blood samples were used for the Fe^{2+}/Fe^{3+} ratio, while the remaining portions were collected into citrate- anticoagulant tubes. Erythrocytes were isolated from whole blood samples after centrifugation at 1,500 X g for 15 minutes using a bench centrifuge followed by careful siphoning of the plasma with Pasteur pipette . The erythrocytes were by repeated inversion, suspended in a volume of isotonic saline equivalent to the siphoned plasma. The suspended plasma was freeze thawed to release a hemolysate used for the hemoglobin polymerization experiment.

2.5. Sickle cell Hemoglobin Polymerization Experiment

The original methods of Iwu, *et al.* [11]; Noguchi and Schechter [12]; Nwaoguikpe, *et al.* [13] were used for HbSS polymerization experiment. Sickle cell haemoglobin polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm using 2 % solution of sodium metabisulphite as a reductant or deoxygenating agent

[11]. 4.4 ml of 2% sodium metabisuphite ($Na_2S_2O_3$). 0.5 ml normal saline (0.9%NaCl) and 0.1 ml hemoglobin were pipetted into a curvette, shaken and absorbance read in a spectrophotometer at 700 nm every 2 minutes for thirty (30) minutes. This represents the control. Distilled water is used as blank for all assays. For the test assay, 4.4 ml of 2 % $Na_2S_2O_3$; 0.5 ml of each extract (antisickling agent) and 0.1 ml (HbSS) solution were pipette into a curvette and readind taken as above. The rates of polymerization were calculated from the formula of average change in absorbance against time in minutes [13]

$Rp = OD_f - OD_i / t$, Rp = Change in OD/ t ;

where= Rp = rate of polymerization;

OD_f = final absorbance, OD_i = initial absorbance

2.6. Determination of Fe^{2+}/Fe^{3+} Ratio

The Fe^{2+}/Fe^{3+} ratio was determined by the methods of Davidson and Henry [14], while the oxygen affinity of hemoglobin and methemoglobin were measured at 540 nm and 630 nm respectively. The approach employs lysing 0.02 ml whole blood in 5.0 ml of distilled water and 0.02 ml normal saline. The absorbance of haemoglobin (Hb) and methemoglobin were measured at 540 nm and 630 nm to determine % Hb and % mHb respectively. This represents the control. To determine the effect of the extracts on Fe^{2+}/Fe^{3+} ratio; 0.02 ml of the antisickling agent was added to 5.0 ml distilled water and 0.02 ml of blood and incubated for 1 hr in a test tube. The absorbance of Hb and metHb were measured according to the method above.

2.7. Determination of Amino Acid Profile of the Samples

The determination of the amino acid profile of the samples was carried out using HPLC equipment consisting of Spectra Physics (San Jose, CA). The equipment comprises of an 8700 XR ternary pump, column heater, injection loop and a software operating system, MS. DOS version 3.2). It involved the preparation of standards and procedures of derivatization and finally, a chromatographic procedure involving gradient elution.

3. Results

Results of all assays are shown in tables 1, 2, 3, 4, 5, 6, 7, 8, 9. Table 1 shows the quantitative phytochemical values of the vegetable extracts expressed in percentage. Table 2 shows the total Ascorbic acid content of the samples expressed in mg/100 g of sample. Table 3 depicts the total free amino acid concentration of the different fractions of the extracts.The fial concentrations expressed in mg/100 g of sample. Table 4 shows the result of hemoglobin polymerization inhibition experiment by extracts of the samples. Table 5 is the result of the Fe^{2+}/Fe^{3+} ratio of HbSS. Table 6 shows the amino acid profile of *Pterocarpus mildbreadi* (Oha) .Table 7 displays the amino acid profile of *Mucuna pruriens* .Table 8 depicts the amino acid profile of *Pterocarpus santalinoides* and table 9 shows the amino acid profile of *Piper methysticum* .

Table-1. Quantitative Phytochemical Content of Samples Expressed in Percent

	Phytochemical %	***Pterocarpus mildbreadi* %**	***Piper methysticum* %**	***Mucuna pruriens* %**	***Pterocarpus santalinoides***
1.	Oxalate	0.255±0.00	0.124±0.00	0.224±0.00	0.168±0.00
2.	Alkaloid	1.470±0.01	4.790±0.00	5.080±0.00	2.330±0.00
3.	Saponin	0.690±0.00	1.010±0.00	1.930±0.00	0.231±0.00
4.	Phytate	0.162±0.00	0.138±±0.00	0.141±0.00	0.139±0.00
5.	Flavonoid	4.050±0.00	8.380±0.00	10.740±0.00	6.410±0.00
6.	Phenol	0.038±0.00	0.176±0.00	0.0.217±0.00	0.154±0.00
7.	Tannin	0.280±0.00	1.230±0.00	1.710±0.00	0.390 ±0.00
8.	Glycoside	0.018±0.00	0.023±0.00	0.190±0.00	0.089±0.00
9.	Terpenoid	3.010±0.00	0.000±0.00	1.001±0.00	6.010±0.00

The results in the table above are the Mean ± SD from triplicate determinations

Table-2. Total Ascorbic acid Content of the Vegetable Samples Expressed in mg/100g of Sample

Sample	**Volume(ml)**	**Conc.(mg/ml)**	**Conc.(mg/10g)**	**Total Conc.(mg/100g)**
Ascorbic Acid (Standard)	85.0	1.70	144.50	1445.0 ±0.00
Mucuna pruriens	84.0	1.30	109.20	1092.0 ±0.00
Pterocarpus mildbreadi	74.0	0.60	44.40	444.0 ±0.00
Pterocarpus santalinoides	85.0	0.70	59.50	595.0±0.00
Piper methysicum	78.0	0.30	23.40	233.3±0.00

The values in the table are the Mean± SD from triplicate determinations

Table-3. Total Free Amino Acid Concentrations of Different Fractions of Samples

Vegetable/Fraction	Volume Extract (ml)	Amino Acid conc.(mg/ml)	Total Conc (mg/10g)	Final Conc.. (mg/100g)
L- Phenylalanine	25.0	20.0	500.0±0.00	5000.0±0.00
Pterocarpus mildbreadi				
FAS	82.0	1.30	106.6±0.00	1066.0±0.00
BUS	28.0	2.00	56.0±0.00	560.0±0.00
WAS	37.0	4.20	155.4±0.00	1554.0±0.00
Piper methysticum				
FAS	79.0	13.20	1042.8±0.00	10,428.0±0.00
BUS	55.0	3.70	203.5±0.00	2035.0±0.00
WAS	77.0	2.50	192.5±0.00	1925.0±0.00
Mucuna pruriens				
FAS	80.0	8.20	656.0±0.00	6560.0±0.00
WAS	67.0	13.70	917.9±0.00	9179.0±0.00
Pterocarpus santaniloides				
FAS	80.0	6.00	480.0±0.00	4800.0±0.00
WAS	59.0	8.00	472.0±0.00	4720.0±0.00

Table-4. Hemoglobin Polymerization Inhibition Experiment of HbSS Blood by Extracts of Samples at $3.7x10^{-1}$ mM Vitamin C Equivalence

Sample	Fraction	Conc. (mM)	Rate of Polymerization	Relative % Polymerization	Relative% Inhibition
HbSS (Control)			0.00077	100±0.00	0.00±0.00
Pterocarpus mildbraedi	CAE	$3.7X10^{-1}$	0.00067	87.01±0.00	12.99±0.00
Piper methysticum	CAE	$3.7X10^{-1}$	0.00033	42.86±0.00	57.14±0.00
Mucuna prurien	CAE	$3.7X10^{-1}$	0.00057	74.03 ±0.00	25.97±0.00
Pterocarpus santalinoides	CAE	$3.7X10^{-1}$	0.00037	48.05 ±0.01	51.95±0.00

The values in the table are the Mean ± SD from triplicate determinations.

Table-5. Results of Fe^{2+}/Fe^{3+} ratio of HbSS at $3.7x\ 10^{-1}$ mM Vitamin C Equivalence

Sample	Fraction	Conc. (% Hb)	Conc. (% mHb)	Fe^{2+}/Fe^{3+}	% Increase/ Decrease
HbSS (Control)			5.91	15.92±0.00	0.00±0.00
Piper methysticum	CAE	93.00	7.00	13.29±0.00	-16.32±0.00
Pterocarpus mildbraedi	CAE	97.08	.92	33.25±0.00	108.86±0.00
Pterocarpus santalinoides	CAE	94.53	5.47	17.28±0.00	8.54±0.00
Mucuna pruriens	CAE	96.56	3.44	28.07±0.00	76.32±0.00

The values in the table are the Mean± SD from triplicate determinations.

Table-6. Total Amino acid Composition of *Pterocarpus mildbraedi* expressed in g/100g of Sample

Amino Acid	Concentration g/100g
Arginine	4.917
Histidine	2.887
Isoleucine	4.100
Leucine	7.723
Lysine	6.315
Methionine	1.372
Phenylalanine	5.156
Threonine	4.250
Tryptophan	1.038
Valine	4.572
Alanine	3.786
Aspartic acid	11.149
Cysteine	1. 286
Glutamic acid	14.462

Glycine	3.355
Proline	4.143
Tyrosine	3.299
Serine	4.470
Asparagine	0.479
Glutamine	0.607

Table-7. Amino acid Composition of *Mucuna pruriens* expressed in g/ 100 g of Sample

Amino Acid	**Concentration g/100g**
Arginine	7.073
Histidine	1.011
Isoleucine	2.984
Leucine	5.052
Lysine	3.455
Methionine	1.080
Phenylalanine	5.240
Threonine	3.790
Tryptophan	8.555
Valine	4.902
Alanine	4.135
Aspartic acid	9.596
Cysteine	2.605
Glutamic acid	15.922
Glycine	3.772
Proline	4.187
Tyrosine	3.036
Serine	4.156
Asparagine	0.413
Glutamine	0.669

Table-8. Total Amino acid Composition of the Extracts of Pterocarpus santalinoides expressed in g/ 100g of Sample

Amino Acids	**Concentration (g/100 g)**
Arginine	6.553
Histidine	3.368
Isoleucine	4.465
Leucine	8.983
Lysine	4.679
Methionine	1.496
Phenylalanine	3.464
Threonine	3.575
Tryptophan	1.046
Valine	4.648
Alanine	6.294
Aspartic acid	9.785
Cysteine	1.436
Glutamic acid	12.565
Glycine	3.648
Proline	1.275
Tyrosine	2.921
Serine	4.331
Asparagine	0.421
Glutamine	0.528

Table-9. Total Amino Acid Composition of *Piper methysticum* expressed in (g/100 g) of Sample

Amino Acid	**Concentration (g/ 100 g)**
Arginine	6.553
Histidine	3.368
Isoleucine	4.603
Leucine	8.983
Lysine	9.720
Methionine	1.496
Phenylalanine	3.911
Threonine	4.339
Tryptophan	1.115

Valine	4.648
Alanine	6.294
Aspartic acid	10.649
Cysteine	1.424
Glutamic acid	14.465
Glycine	4.212
Proline	3.027
Tyrosine	2.921
Serine	4.331
Asparagine	0.458
Glutamine	0.608

4. Discussion

The antisickling potentials of four vegetables extracts (*P. mildbraedi, P.santalinoides, M. pruriens* and *P. methysticum*) were investigated to ascertain the ability of the extracts to inhibit sickle cell haemoglobin polymerization and improve the oxidant status(Fe 2+/Fe 3+ ratio of sickle cell blood . The various vegetable extracts were analysed for their bioactive principles which revealed rich sources of phytochemicals shown in table 1.0. The table shows high preponderanc of phytonutrients like flavonoids, alkaloids, saponins, tannins, phenols and others. Phytochemicals are very abundant in the various vegetative species such as flavonoid with the various compositions as in *P. mildbraedi* (4.05%), *P. methysticum* (8.380%), *M. pruriens* (10.74%) and *P. santalinoides* (6.450%) . Table 2 shows the ascorbic acid concentration of the extracts expressed in mg/ 100g of sample. The vegetable samples are very rich sources of this antioxidant vitamin which had earlier been shown by some workers to be antisickling [15]. Vitamin C has been implicated in various researches on the management of sickle cell disease because of its role as an antioxidant. Among the various antioxidants, flavonoids are naturally occurring phenolic compounds in plants. In this work as shown in table 1, the antioxidants are really abundant in these vegetable samples to qualify them play the role as antisickling agents.

Vitamin C, β-carotene, and Vitamin E are all powerful antioxidants [16]. Studies indicate that vitamin-mineral supplements (Vit. C, E, zinc and magnesium) have been effectivel used at high doses to reduce the percentage of irreversibly sickle cells (ISC) in (SCD) patients [17-20]. The role of antioxidants especially in various antisickling agents has engendered our interest to look at this group of compounds for a solution to this debilitating syndrome. It is believed that the higher the antioxidant property of an antisickling agent, the higher its possible antisickling effect as this enables it to reduce oxidative stress that contribute to sickle cell crisis.

The role of amino acids in sickle cell disease management cannot be under-rated. Amino acids apart from being necessary in nutrition have also been found as antisicking agents. Such amino acids as Phenylalanine, Lysine, Glutamine, Arginine, Asparagine and others, have been found to elicit haemoglobin polymerization inhibition at the heme pocket and improve the Fe^{2+}/Fe^{3+}ratio, [12, 21-23]. The vegetable extracts under survey are found to good sources of amino acids both for nutrition and for antisickling effectiveness. Tables 6, 7, 8, and 9, show the amino acid profiles of the extracts. The results of haemoglobin polymerization inhibition experiment and Fe^{2+}/Fe^{3+} ratio on table 4 and 5; show that the extracts of the vegetable samples inhibited polymerization remarkably with *P. methysticum* and *P. santalinoides* being the best. Fe^{2+}/ Fe^{3+} ratio is a measure of improvement of the oxidant status of sickle erythrocytes in the presence of antisickling agent. It also measures the conversion of metHb to Hb. Table 5 shows this effect and it can be seen that almost all samples improved this status with *Pteracarpus mildbraedi* eliciting the highest response with an increase of 108% , followed by *M.pruriens*. *P. methysticum* did not improve this ratio despite the fact that it inhibited hemoglobin polymerization to about 57.14%.. This explains that many antisicking agents possess different mechanisms of antisickling effectiveness or action.

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