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Full Length Research Paper

Polymerization inhibition activity of *Raphia hookeri* palm sap and its effect on osmotic fragility of sickle cell red blood cells

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The influence of *Raphia hookeri* palm sap as an antipolymerization agent of sickle cell haemoglobin (HbSS) and its effect on the osmotic fragility of sickle cell anaemia (SCA) red blood cells (RBC's), *in vitro*, were investigated. Phytochemicals detected in the palm sap included tannins, flavonoids, saponins, thiocyanates, cyanogenic glycosides, alkaloids and catechins. The chemical constituents included 4-hydroxybenzoic acid (4-HBA), vitamin C, free amino acids and monosaccharides. The levels (in g/ 100 g protein) of four antipolymerization/ antisickling amino acids in the palm sap were phenylalanine (4.20±0.07), leucine (2.05±0.07), arginine (3.22±0.08) and valine (4.38±0.09). Minerals evaluated were in parts per million (ppm) in the palm sap included potassium ion (K^+) (18.00), sodium ion (Na^+) (26.00), magnesium (II) ion (Mg^{2+}) (19.20), calcium (II) ion (Ca^{2+}) (101.20), ion (II) ion (Fe^{2+}) (26.00) and zinc (II) ion (Zn^{2+}) (16.80). The palm sap inhibited the polymerization of HbSS and reduced the osmotic lyses of its erythrocytes, *in vitro*. The bioactive antipolymerization agents detected in it were 4-HBA, flavonoids, thiocyanates and amino acids like phenylalanine, leucine, arginine and valine. In conclusion, the study established the antipolymerization potential of *R. hookeri* sap, established that it reduced osmotic lyses of SCA RBC's and identified the bioactive agents in the palm sap.

Key words: Antipolymerization, fragility, *Raphia hookeri* sap, sickle cell.

INTRODUCTION

The homozygous state or sickle cell anaemia (SCA) causes moderate to severe haemolytic anaemia. The main clinical disability from repeated episodes of vascular occlusion by sickle red blood cell results in acute crises and eventual end – organ damage. The clinical severity of SCA is variable. This is partly due to the effect of inherited modifying factors, such as interaction with β-thalassaemia or increased synthesis of foetal haemoglobin (HbF) and partly due to socio-economic conditions and other factors that influence general health (Wild and Bain, 2002). SCA is the most lethal type of sickle-cell disease (SCD) (Uzoegwu and Onwurah, 2003). SCD is a collective name for a group of conditions characterized by the formation of sickle red blood cells (Wild and Bain, 2002). Pre-disposition to SCA results from the prevalence of malaria and the sickle-cell trait (HbAS) (Armstrong, 1983). Polymerization of sickle haemoglobin is the catalyst in the development of vaso-occlusion (Claster and Vichinsky, 2003). The patho-genesis of SCA has centered on the sequence of events that occur between polymerization of deoxy-haemoglobin S and vaso-occlusion. Cellular dehydration, inflammatory response and reperfusion injury seem to be important pathophysiological mechanisms (Ballas, 2002).

Palm wine is a popular traditional alcoholic beverage consumed by more than 10 million people in West Africa (Ukhun et al., 2005). It is consumed throughout the tropics and appears as a whitish liquid produced by natural fermentation of the sap of *Elaeis guineensis* and *Raphia hookeri*. Palm wine is essentially a heavy suspension of yeasts and bacteria in fermenting palm sap

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(Okafor, 1975). The unfermented sap is a clean, sweet and colourless syrup containing sugar, which is mainly sucrose. Palm sap is given widely to children possibly as a result of its nutritional value and the general belief about the absence or negligible alcohol content (Omigbodun and Babalola, 2004). It is a good source of vitamins B_1 (thiamin), C (ascorbic acid) and offers supplemental nutrition to a meal. The drink is a rich nutrient medium containing sugars, proteins, amino acids, alcohols and minerals (Ukhun et al., 2005). Palm wine can be consumed in a variety of flavours varying from sweet 'unfermented' to sour fermented and vinegary alcoholic drinks. The fermentation process increases the level of thiamin, riboflavin, pyridoxine and vitamin B_{12}. *Saccharomyces cerevisiae* is able to concentrate large quantities of thiamin, nicotinic acid and biotin and thus form enriched products. Palm wine in West Africa is high in vitamins B_{12}, which is very important for people with low meat intake and who subsist primarily on vegetarian diets (FAO, 1998; Ezeagu and Fafunso, 2003). The raffia palm (R*. hookeri)* is commonly found in West Africa and in abundance particularly in southeastern Nigeria. It usually grows up to 12 m high (Akpan and Usoh, 2004). The root extract is used in traditional medicine for the treatment and prevention of several diseases; it is given to infants with stomach pain (Akpan et al., 1996). Again, Nigeria is the most populous country in Africa and is known to have the highest number of SCD patients in the world (Uzoegwu and Onwurah, 2003). The challenge at present is to improve the quality of health of the increasing number of patients with SCA, especially with the attendant poverty level. So, such source(s) of treatment must have the qualities of being affordable and available. Raffia palm sap may have such qualities. This paper presents the sickle cell haemoglobin (HbSS) polymerization inhibition activity of *R. hookeri* palm sap and its effect on the osmotic fragility of SCA erythrocytes.

MATERIALS AND METHODS

Procurement and preparations of palm sap and blood specimen

The palm sap that was used was tapped from a *R. hookeri* G. Mann. and H. Wendl. palm tree by a palm wine tapper at Orodo, Mbaitoli local government area of Imo state Nigeria.

Homogenous solution of the palm sap was obtained by filtering it through a Whatman 24 filter paper. The palm sap was heated to 85°C [to halt fermentation and drive off pre-formed ethanol (b.p 78°C)], then cooled. Nwaiwu et al. (2005) reported that yeast cells are damaged at temperatures of 80°C and above.

Blood specimen was collected with informed consent by venipuncture from a healthy homozygous (HbSS) donor. The blood sample was put in a heparinized sequestering bottle and used within 24 h. Four drops of the blood specimen was washed thrice in normal saline and the red blood cells (RBCs) lysed in distilled water, to produce the haemoglobin (Hb) solution.

Analyses of palm sap

The presence of 4-hydroxybenzoic acid (4-HBA) was detected using the method of ASEAN (2005) which employed the use of Millon's reagent. An aspect of the method used for the estimation of tannins by AOAC (1984) was adopted for the detection of tannins: 1.0 ml of the palm sap was mixed with 0.5 ml of Folin - Denis reagent and 1.0 ml of 17% sodium carbonate (Na_2CO_3). The mixture was left to stand at room temperature (30°C) for 3 min for a blue colour development. Test for the presence of catechins, β-carotene, cardiac glycosides, flavonoids and alkaloids were carried out using the methods of Evans (2002). Test for the presence of cyanogenic glycosides was done using the method of AOAC (1990). Test for the presence of saponins was carried out using the method of Sofowora (2006). Test for the presence of thiocyanates was carried out by modifying the alkaline picrate paper method of Haque and Bradbury (1999). The modification made was that the resulting orange/ brick-red paper strip was not washed in distilled water and the colour intensity measured spectrophotometrically at 510 nm. Test for the presence of vitamin C was evaluated using the method of Lambert and Muir (1968). The presence of free amino acids and monosaccharides were detected using the methods of Plummer (1971). The profile of some of the amino acids was carried out using the technicon sequential multi-sample (TSM) amino acid analyzer (model DNA 0209) and chromatographic methods described by Spackman et al. (1958). The mineral contents were estimated using the methods of Allen et al. (1983) and AOAC (1990).

In vitro polymerization assay

The method of Nwaoguikpe and Uwakwe (2005) was modified by monitoring the turbidity at 555 nm instead of 700 nm [Hb solution absorb maximally at 555 nm (Plummer, 1971)]. 2.0 ml of freshly prepared 2% sodium metabisulfite ($Na_2S_2O_5$), 2.0 ml of normal saline and 0.1 ml of the HbSS solution were pipetted into a cuvette, shaken and the polymerization of HbSS monitored using a digital spectrophotometer [model 590 (Turner®, USA)] for 30 min. This served as the control.

The standard assays were respectively run using 4-hydroxybenzoic acid (5 mg/ml) and 0.05% quercetin in place of normal saline, while the test assay was run using the homogenous palm sap instead of normal saline. Rate of polymerization (ROP) per minute was calculated by dividing the difference between the final absorbance reading and the initial absorbance reading by the total time of assay (30 min). Relative percentage polymerization (RPP) was calculated by assigning a value of 100% to the control and those of the others calculated by comparing their ROP values relative to the ROP of the control. Percentage polymerization inhibition (PPI) was calculated by subtracting the value of RPP from 100%.

Osmotic fragility assay

The osmotic fragility test was based on a modification of the method of Roper et al. (2002). The modification made was that 1.0 ml of the final 5.0 ml that was taken into the test tubes before the addition of the heparinized blood was replaced with 1.0 ml of the palm sap. Plots of percentage lysis versus concentration of sodium chloride (NaCl) (g/l) were made, and their mean corpuscular fragility (MCF) values at 50% erythrocytes lyses extrapolated.

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA)

Table 1. Phytochemical and chemical constituents of the palm sap*.

Parameter	Result
4-HBA	+
Tannins	+
Flavonoids	+
Saponins	+
Vitamin C	+
β-carotene	-
Catechins	+
Thiocyanates	+
Cyanogenic glycosides	+
Cardiac glycosides	+
Alkaloids	-
Amino acids	+
Monosacchrides	+

*Values are means of triplicate determinations. **+,** Present; **-**, absent.

Table 2. Amino acid profile of the palm sap (g/100 g protein)*.

Amino acid	Result
Phe	4.20 ± 0.07^{a}
Leu	2.05 ± 0.07^{c}
Val	4.38 ± 0.09^{a}
Arg	3.22 ± 0.08^{t}

*Values are means of duplicate determinations. Values on the same column with the same superscript letter are not significantly different at (P<0.05).

and students' t-test of significance at (P<0.05).

RESULTS AND DISCUSSION

Most of the phytochemicals and chemicals detected in the palm sap (Table 1) were also detected by Akpan and Usoh (2004) in the aqueous root extract of *R.hookeri*. The antisickling effects of thiocyanates, 4-HBA and the antisickling amino acids presented in Tables 1 and 2 have been reported by Gorecki et al. (1980), Acquaye et al. (1982), Armstrong (1983), Balagopalan et al. (1988) and Oyewole et al. (2008). Onah et al. (2002) also reported the presence of free amino acids, phenolic compounds, tannins, globulins and saponins in the aqueous-methanolic extract of *Cajanus cajan* seed which they found to have had antisickling potentials. The nutritional importance of the minerals embodied in Table 3 are widely known; especially in the ATPase systems (for potassium ion (K^+), sodium ion (Na^+), magnesium (II) ion(Mg^{2+}) and calcium (II) ion (Ca^{2+})), red blood cell synthesis (for ion (II) ion (Fe^{2+}) and zinc (II) ion (Zn^{2+})), muscular contraction, growth, maintenance of cellular fluid balance (Mg^{2+}, K^+ and Na^+) and immunologic responses (Cooper, 2000; Nelson and Cox, 2000; Wardlaw and Kessel, 2002; Chaney, 2006). The roles of K^+, Na^+, Mg^{2+} and Ca^{2+} in the pathophysiology of HbSS erythrocytes have been reported by Brugnara (1995) and Cotran et al. (1999). Magnesium causes a rehydration of RBC's (Brugnara, 1995). The fear of a possible iron overload and its induction of oxidative stress may be allayed when one is reminded that the eventual absorption of iron (non-haem iron) from the body store (ferritin) depends on the body's requirement. Vitamin C (also detected in the sap) encourages the uptake of non-haem iron (Fe^{3+}) by reducing it to the ferrous state; the absorbed iron would still be stored in ferritin until required by the body. Polyphenols (like tannins) decrease iron absorption while calcium inhibits it (absorption).

Iron absorption is also regulated by the mucosal block where ferritin stores are either later absorbed or sloughed off into the gastrointestinal tract with the intestinal cell after 2 to 5 days cycle and excreted. There is substantial evidence that zinc supplementation may reduce the impact of many diseases such as sickle-cell disease

Table 3. Mineral contents of the palm sap (ppm)*.

Sample	K^+	Na^+	Mg^{2+}	Ca^{2+}	Fe^{2+}	Zn^{2+}
Palm sap	18.000^d ±0.001	26.000^b ±0.003	19.200^c ±0.004	101.200^a ±0.003	26.000^b ±0.002	16.800^m ±0.003

*Values are means ± S.D of triplicate determinations. Values on the same row with the same superscript letter are not significantly different at ($P<0.05$).

Table 4. Effects on ROP, RPP and RPI values.

Sample	ROP	RPP	PPI
Control	2.13×10^{-2}	100.00	0.00
Palm sap	7.0×10^{-3}	32.86	67.14
4-HBA	0.00	0.00	100.00
Quercetin	0.00	0.00	100.00

Table 5. Results of the *in vitro* osmotic fragility tests with the palm sap.

Sample	MCF (NaCl) g/l*
HbSS (control)	3.130±0.002
With palm sap	2.870±0.001

*Values are means ± S.D of triplicate determinations.

(Wardlaw and Kessel, 2002).

Table 4 was run with HbSS solution indicating that there must have been direct interactions with the HbSS molecules. The antisickling moiety, 4-HBA, may have bound to tryptophan (Trp) residues of Hb, causing conformational changes in the deoxy-Hb structure. Olaniyi (1989) reported that 4-HBA bound to Trp residues of albumin through the benzenoid rings of its aromatic side chain and the flat ring of the albumin's tryptophan residue by van der Waals forces. Garrett and Grisham (1999) reported that Trp existed as α15, β15 and β39 residues of the Hb primary structure. Quercetin (flavonoids), being an aromatic compound, may have intercalated at or near the haem pockets in the α and β chains of the deoxy-HbS. Russu et al. (1986) said that antisickling agents bound competitively at hydrophobic pockets at or near the β6 position containing the mutation in HbSS molecules thereby inhibiting gelation. Flavonoids may also have bound to the Trp residues of deoxy-Hb (just like 4-HBA), causing conformational changes in the deoxy-HbSS molecule. Thiocyanates bind by covalently carbamoylating the termini valine amino acid residues of the β-chains and particularly those of the α-chains in the presence of 2, 3-bisphosphoglycerate (2, 3-BPG) which cause conformational changes in the local deoxy-Hb structure that increase its oxygen affinity and decrease gelation (Chang et al., 1983). These may have reduced the concentration of polymerizable deoxy-Hb. On the other hand, the antisickling amino acids reduce hydrophobic interactions between deoxy-HbS by competitively binding to hydrophobic sites with the substituted valine at β6 position of one deoxy-Hb molecule, on one hand and leucine and phenylalanine at β88 and β85 positions, respectively, of an adjacent polymerizable deoxy-Hb (Armstrong, 1983). It would appear that the antipolymerization mechanisms exhibited by the palm sap were both competitive and allosteric; their actions probably being additive.

The palm sap also reduced the osmotic lyses of HbSS erythrocytes (Table 5), shifting the fragiliogram curve to the left. The decrease in the osmotic lyses of the RBCs observed here did not mean that the RBCs were unusually flattened (leptocytes); in which the volume to surface area ratio was decreased or that they had low mean corpuscular Hb (MCH) and mean corpuscular volume (MCV). This decrease may have been due to the contributory effects of its monosaccharides (fructose and glucose). Roper et al. (2002) reported that during incubation, the metabolism of the red blood cell normally became stressed and the pumping mechanisms tended to fail; with the relative lack of glucose in the medium as one of the factor. The monosaccharides in the palm sap

may have provided the energy, and later reduced nicotinamide adenine dinucleotide phosphate (NADPH) through the pentose phosphate pathway, with which they resisted lyses while the erythrocytes were under incubation. Harris (2006) reported that glucose in erythrocytes provided energy in the form of adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide phosphate (NADPH) required for the reduction of glutathione that was used in the destruction of organic peroxides and hydrogen peroxide (H_2O_2) which caused irreversible damage to membranes, deoxyribonucleic acid (DNA) and other cellular components. This reduction in osmotic lyses of HbSS erythrocytes would suggest increase in their life spans. The palm sap may equally have engendered isotonicity which does not encourage haemolysis, since it they contained solutes as indicated in Tables 1, 2 and 3. Many of the phytochemicals, chemical and mineral constituents like flavonoids, amino acids, glucose, K^+ and Mg^{2+} are also important in maintaining red blood cell rheology (Acquaye et al., 1982; Brugnara, 1995; Cesquini et al., 2003; Harris, 2006). Some of these phytochemicals and macronutrients were also detected by Imaga et al. (2010) in the aqueous-methanolic extracts of the leaf and stem of *Parquetina nigrescens* L. which exhibited both inhibitory action on HbSS RBC membrane lyses and antisickling potential.

Conclusion

The study showed that the palm sap could be used as a medicine in the treatment of SCD.

Abbreviations: HbSS, Sickle cell haemoglobin; **SCA,** sickle cell anaemia; **RBCs,** red blood cells; 4-**HBA,** 4-hydroxybenzoic acid; **PPM,** parts per million; **HbF,** foetal haemoglobin; **SCD,** sickle-cell disease; **HbAS**, sickle-cell trait; **b.p,** boiling point; **Na_2CO_3,** sodium carbonate; **TSM,** technicon sequential multi-sample; **$Na_2S_2O_5$,** sodium metabisulfite; **ROP,** rate of polymerization; **RPP,** relative percentage polymerization; **PPI,** percentage polymerization inhibition; **NaCl,** sodium chloride; **MCF,** mean corpuscular fragility; **Trp,** tryptophan; **Hb,** haemoglobin; **2, 3-BPG,** 2, 3-bisphosphoglycerate; **MCH,** mean corpuscular Hb; **MCV,** mean corpuscular volume; **NADPH,** nicotinamide adenine dinucleotide phosphate; **ATP,** adenosine triphosphate; **DNA,** deoxyribonucleic acid; **H_2O_2,** hydrogen peroxide; **K^+,** potassium ion; **Na^+,** sodium ion; **Ca^{2+},** calcium (II) ion; **Mg^{2+},** magnesium (II) ion; **Zn^{2+},** zinc (II) ion; **Fe^{2+},** ion (II) ion.

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