

Full Length Research Paper

Antisickling property of *Carica papaya* leaf extract

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Sickle cell disease (SCD) results from a mutation in the hemoglobin inside the red blood cells, where a glutamic acid at position 6 is replaced by a valine. Many phytomedicines have been identified as potential antisickling agents, stemming from reported usage as ethnomedicines by the local folk. This research examined methanolic leaf extracts of *Carica papaya* L. (Caricaceae) for possible *in vitro* antisickling and membrane-stabilizing activities involving the use of positive (p-hydroxybenzoic acid 5 µg/ml) and negative (normal saline) controls for the antisickling experiments and osmotic fragility test on Hb^{ss} red blood cells obtained from non-crisis state sickle cell patients. Fragiliograms indicated that the plant extract reduced hemolysis and protected erythrocyte membrane integrity under osmotic stress conditions. Pretreatment of SS cell suspensions with *C. papaya* leaf extract inhibited formation of sickle cells under severe hypoxia, with only 0 - 5% sickle cells at 40 min compared with untreated SS cell suspensions which had over 60% sickle cells. These results indicate the feasibility of *C. papaya* as an attractive potential candidate for SCD therapy.

Key words: Antisickling, membrane-stabilizing, sickle cell disease, erythrocyte fragility, *Carica papaya*, toxicity profile.

INTRODUCTION

Sickled erythrocytes tend to block capillaries *in vivo*, causing stasis and thereby starve organs of both nutrient and oxygen leading to organ damage. *In vitro* studies have shown that some antisickling agents, especially those of plant origin, affect the kinetics of hemoglobin (Hb) polymerization and inhibit the time course for red blood cell sickling (Iyamu et al., 2003, 2004). A characteristic property of the gelation of deoxy-HbS is the existence of a delay time prior to polymerization of deoxy-HbS molecules]. It is believed that the delay time represents the time required for the formation of nuclei. A drug that prolongs the delay time prior to polymerization might be of therapeutic value in SCD, because a longer delay time decreases the probability of sickling of HbS red

blood cells. Reported antisickling agents in this group include Niprisan, MX-1520 and 5HMF (Iyamu et al., 2003; Chaojie Zhang et al., 2004; Abdulmalik et al., 2005), which modify intracellular sickle hemoglobin and inhibit sickling of red blood cells. Attempts to find alternative, cheaper and less toxic therapies for SCD management, led to the discovery of antisickling properties of *Cajanus cajan* seeds (Ekeke and Shode, 1985) and *Zanthoxylum macrophylla* (formerly *Fagara*) roots used locally by traditional healers in Nigeria (Sofowora et al., 1975; Elekwa et al., 2005).

Fagara (otherwise called "orin-ata") roots have been analyzed for antiprotease and membrane stabilizing activity using a modified osmotic fragility technique to analyze membrane stabilization action (Oyedapo and Famurewa, 1995). It has been discovered that the antisickling (and anti-inflammatory) action of *Fagara* was due to its o-hydroxybenzoic acid constituent (Sofowora et al., 1975).

Other plants purported to be used as herbal therapies for SCD include *Parquetina nigrescens* root extracts (Kade et al., 2003) and *Carica papaya* leaves (folk medicine reports). Aqueous and ethanolic extracts of several

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Abbreviations: AST, Aspartate serum transaminase; Hb^{ss}, sickle hemoglobin; ALT, alanine serum transaminase; SCD, sickle cell disorder; Hb, hemoglobin.

phytochemicals have been evaluated for significant *in vitro* antisickling activity. Recent studies support the claims of traditional healers and suggest a possible correlation between the chemical composition of these plants and their uses in traditional medicine (Mpiana et al., 2007).

The antisickling and erythrocyte membrane stabilizing activities of *C. papaya* L. (Caricaceae) as well as its toxicity profile were investigated in this study.

MATERIALS AND METHODS

Blood samples, chemicals and biochemicals

Fresh blood samples were collected with full informed consent from sickle cell individuals in the steady state of the disease aged between 16 and 20 years of both sexes, who had not taken any herbal medication for SCD during routine visits at the Sickle Cell Out Patients' Clinic of the Lagos University Teaching Hospital, Idi-Araba, Lagos, Nigeria. 5 ml venous blood samples were collected in sodium EDTA bottles and used for osmotic fragility and antisickling tests.

Diagnostic test kits for AST, ALT and creatinine were purchased from RANDOX Company Limited, UK and the assays performed according to manufacturer's instructions. Unless stated otherwise, all other chemicals used were of analytical grade obtained from Sigma Chemical Company and used without further purification.

Plant material

Leaves of *C. papaya* L. were collected from the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria and identified by Mr. Usang Felix of the Institute. A Voucher Specimen (FHI: 106994) is deposited at the herbarium in FRIN.

Extraction of the leaves

Dried leaves of *C. papaya* L. were ground in a cross beater mill equipped with a 1 mm sieve. An aliquot (400 g) was extracted by exhaustive soxhlet extraction in a method described earlier (Ogoda et al., 2002) with petroleum ether 60 - 80°C and aqueous - methanol (1:3, 60 - 80°C) as solvents. The extracts were stored at 4°C in freeze - dried form and used for the antisickling experiments, osmotic fragility test and toxicological assessment.

Proximate analysis

The procedure of Horwitz (2000) was employed for the determination of the percentage proximate composition of carbohydrate, lipid, protein and other nutrients of the dry ground leaves of *C. papaya* L.

Phytochemical screening

The procedure of Trease and Evans (1996) was used to screen *C. papaya* leaves for the presence of phenolic compounds, tannins, saponins and glycosides.

Osmotic fragility tests

The osmotic fragility of erythrocytes measures the membrane stabilizing effect of the extracts in osmotic stress/ hypotonic lysis after 30

min incubation. The protocol by Jaja et al. (2000) was used for the analysis, with some modifications. To 10 ml reaction vessels containing 4 ml of different concentrations (0.00 - 0.85%) of buffered saline pH 7.4, 1 ml of a range of concentrations of extract (1 mg/ml to 5 mg/ml) and 0.05 ml Hb^{ss} blood were added. The mixture was left to incubate at room temperature for 30 mins and then centrifuged at 2000 rpm for 15 mins. The supernatant was collected and read at 540 nm against blank (0.85% buffered saline concentration).

Antisickling activity

The homozygous SS blood samples obtained from patients were washed thrice in phosphate buffered saline to obtain the red blood cells which were then resuspended in normal saline and used for the analysis according to earlier described methods of Acquaye et al. (1982), Ekeke et al. (1990) and Ogoda et al. (2002). The aqueous and methanol extracts of *C. papaya* leaves were used in this experiment, with para-hydroxybenzoic acid as the chemical standard. 1 ml SS blood cell suspensions were pre-incubated with 0 to 10 mg/ml concentrations of the extracts in the presence of 2% sodium metabisulphite solution and then microscopic analysis of the time course of the effect of varied concentrations of extracts on the sickling of SS erythrocytes was done. A plot of percentage sickling inhibition against extract concentration was analyzed for possible explanation of the observed antisickling effect.

Toxicity assessments

Experimental rats were obtained from the animal house in the College of Medicine, University of Lagos, Nigeria and handled with utmost care under the approval of the local ethical committee throughout the duration of the experiments. Forty albino rats weighing 180 - 225 g were divided into 4 groups and administered various doses (2000 - 16, 000 mg/kg/d) of *C. papaya* extract once, by gavage. An equivalent volume of water was administered by gavage to control rat. The rats were observed for 24 h and thereafter for 14 days to ascertain that there was no delayed toxicity. At the end of all experiments, all surviving rats were euthanized by cervical dislocation under anesthesia and used for biochemical and histopathology analyses; serum enzymes and metabolites indicative of liver and kidney functions were determined using blood samples from the rats, following the manufacturer's instructions as contained in the RANDOX diagnostics test kits for transaminase (AST and ALT) and creatinine. Tissue samples of the organs put in 10% phosphate - buffered formaldehyde (Iyamu et al., 2003) were embedded in paraffin according to standard methods. Sections were cut and stained by a haematoxylin - eosin (HE) solution for light microscopy.

Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). The student's t-test was used for comparison of the experimental groups. The level of significance was set at $p < 0.05$.

RESULTS

The aqueous - methanolic extract had a 5.78% yield. *C. papaya* leaf extract was found to contain alkaloids, flavonoids, glycosides, cardiac glycosides, tannins, saponins and anthraquinones. Proximate analysis of the plants showed that all the macronutrients were present with car-

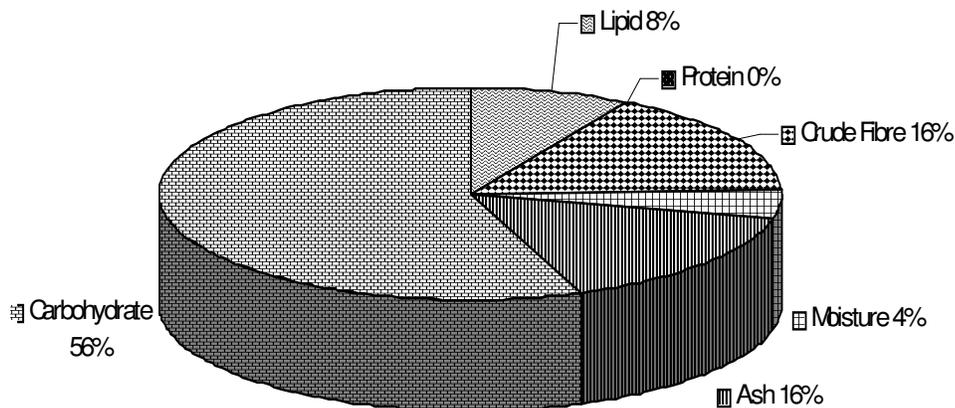


Figure 1. Percentage proximate composition of *C. papaya* leaves.

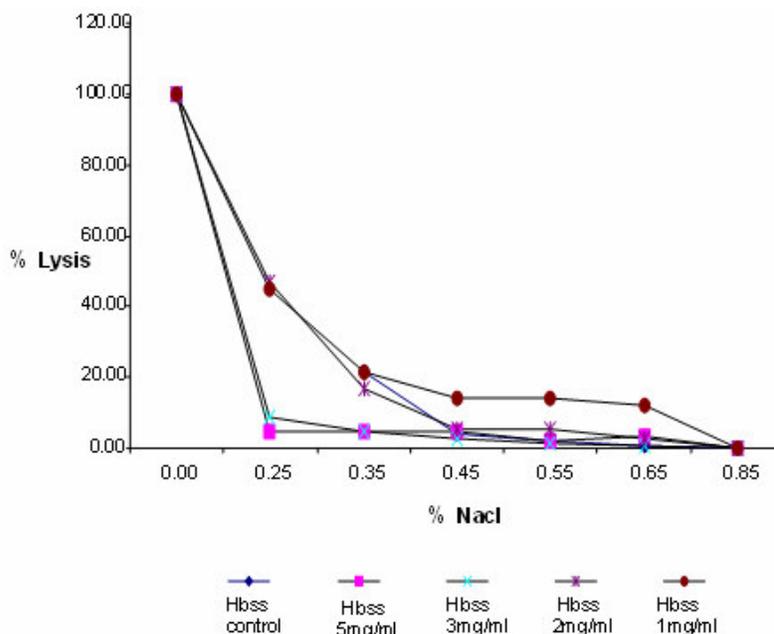


Figure 2. Osmotic fragility after supplementation with various concentrations of *C. papaya* extracts. The different extracts of *C. papaya* gave similar results as shown. The 3 mg/ml extract was most stable, reducing RBC lysis at 0.25% buffered saline concentration to about 5% compared with 50% of that of the control. Tailing on the x axis (indicative of irreversibly sickled cells (ISC) present) was also reduced.

bohydrate being the most abundant (Figure 1). Figure 2 shows the osmotic fragility before and after supplementation with varied concentrations of papaya extract. Fragility showed a marked decrease in percentage hemolysis for the 3 mg/ml extract (100 - 8%) at 0.25% buffered saline concentration. In the biochemical analysis, serum transaminase levels were slightly higher in all the experimental groups after day 14. Creatinine levels were significantly higher in the 2000 mg group with $134.65 \pm 2.80 \mu\text{mol/l}$ ($p < 0.05$) compared with the control which was $70.95 \mu\text{mol/l}$. Histological examination of the

organs did not show any significant pathological alterations in the organs of both control and test groups.

Data from *in vitro* studies on the antisickling activity of the herbal extract carried out on blood samples collected from confirmed non-crisis sickle cell individuals, show that pretreatment of SS cell suspensions with *C. papaya* leaf extract inhibited the formation of sickle cells under severe hypoxia, with only 0 - 2% sickle cells at 40min compared with untreated SS cell suspensions which had over 60% sickle cells compared with the controls (Figure 3).

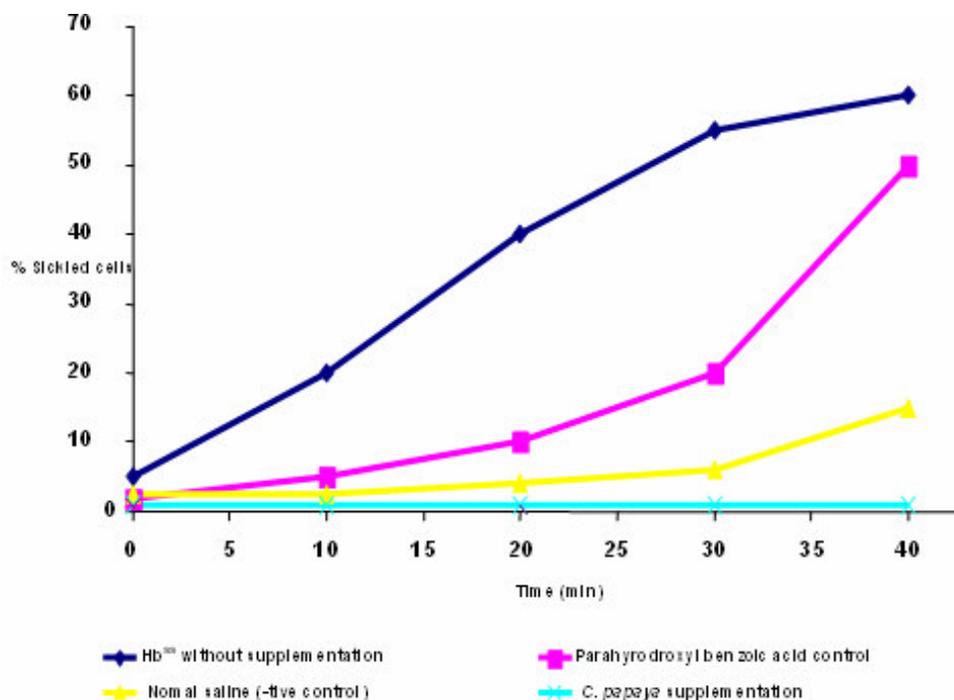


Figure 3. *In vitro* antisickling activity of 5 mg/ml concentrations of *C. papaya* extracts. This represents data obtained from a typical three independent experiments performed in duplicate using blood samples from three SS patients. Sickle cell suspensions were preincubated with extracts prior to exposure to 2% sodium metabisulphite solution: as shown, the time course for 60% sickling was 40 min for the control (blood without supplementation). However, *C. papaya* (5 mg/ml) reduced sickling to 0% as shown.

DISCUSSION

Research into phytotherapy of diseases is a current trend in the management of tropical diseases and genetic disorders like sickle cell anemia, with a view to finding cheaper, alternative medicines that the wide populace can have immediate access to. Recent studies show that unripe papaya fruit extract has antisickling activity (Oduola et al., 2006). In this study *C. papaya* leaf extract was found to have an appreciable potent antisickling activity and greatly affected the time course for sickling in a dose-dependent manner, the most effective doses being 5 and 10 mg/ml extract concentrations. Antisickling agents have been reported to prolong delay time of Hb polymerization as part of the mechanisms for its antisickling action (Iyamu et al., 2002). *C. papaya* leaf extract was not found to prolong the delay time in this study but definitely inhibited HbSS polymerization indicating that the extract may apply a target hit on HbS polymerization in attenuating SS cell sickling. This could also indicate that the effect of the extract is probably at the cell membrane level and not direct interaction with HbS molecules unlike other reported compounds (Abdulmalik et al., 2005; Iyamu et al., 2003), whose antisickling actions are premised on the interaction with HbS molecules.

The effect of varied concentrations of the *C. papaya* extracts on erythrocyte membranes was analyzed using the

osmotic fragility test, which revealed appreciable membrane protective effects of the herb and its inhibitory action on the hemolysis of red blood cells.

The toxicity profile of the plant as assessed by histological and biochemical analyses did not reveal any substantial toxicity of the plant. Patients given this herb will benefit from its total inhibition of the sickling phenomenon at low dose concentrations. These results indicate the feasibility of *C. papaya* leaf extract as an attractive potential candidate for SCD therapy and strongly collaborates the ethnomedical usage of the plant.

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