Anti-Sickling and Membrane Stabilizing Effects of Carica papaya Leaf Extract

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Authors’ contributions

This work was carried out in collaboration between all authors. Author AON designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author BCO managed the literature searches and edited the manuscript. Author CO carried out the analyses of the study and the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

The antisickling and membrane stabilizing effect of aqueous extract of Carica papaya leaf was investigated. Fifty confirmed HbSS patients on routine clinical checkup were involved in this experiment after their consent has been obtained. 50 samples of HbSS blood were collected. The samples were divided into five groups, with ten (10) samples in each group. The samples in the different groups were treated with different concentration of the extract ranging from 2mg/ml to 10mg/ml. In group 1 the samples were treated with 2mg/ml concentration of Carica papaya leaf extract. In group 2 the samples were treated with 4mg/ml concentration of Carica papaya leaf extract. In group 3 the samples were treated with 6mg/ml concentration of Carica papaya leaf extract. Group 4 samples were treated with 8mg/ml concentration of Carica papaya leaf extract. Group 5 samples were treated with 10mg/ml concentration of Carica papaya leaf extract. For each sample three different tests were carried out (osmotic fragility, sickling test and reversal sickling test). For each test, two test tubes were set up; control without Carica papaya leaf extract and Experiment with Carica papaya leaf extract. Data obtained was analysed using Student’s T-test on SPSS software computer package. Results showed a dose dependent significant reduction (p<0.05) in sickling of Reb Blood Cell treated with different doses of the extract, also the extract...
1.1 Background of Study

Sickle cell disease (SCD) is known to be one of the diseases wrecking most parts of the globe without any discrimination of ethnic or racial standards [1]. It is characterized by a variety of symptoms including shortness of breath, heart palpitations, abdominal pains, aches and pains in the muscle [2]. The crisis stage is characterized by severe pain in the head and whole body. In the crisis stage, if the percentage of sickled erythrocytes can be lowered we can expect a great relief to the patient and this could be a great step towards the management of SCD [1]. SCD is an autosomal recessive genetic blood disorder characterized by red blood cells that assume an abnormal, rigid, sickle shape. This disease is chronic, lifelong and it is predominant in people of African, Mediterranean, Indian and middle Eastern descent as well as blacks and Hispanics in the Caribbean and Central America [2]. SCD is caused by point mutation in the beta globin chain of hemoglobin (Hb) causing the hydrophilic amino acid glutamic acid to be replaced with the hydrophobic amino acid valine at the sixth position. The beta globin gene is found on the chromosome 11 [3]. Under low oxygen conditions, the absence of a polar amino acid at position six of the beta globin chain, promotes the non-covalent polymerisation (aggregation) of hemoglobin, which distorts red blood cells into a sickle shape and decreases their elasticity and the loss of RBC elasticity is central to the pathophysiology of SCD.

Normal RBCs are quite elastic, which allows the cells to deform to pass through capillaries. However repeated episodes of sickling damage the cell membrane and decrease the cells elasticity, as a consequence, these rigid blood cells are unable to deform as they pass through narrow capillaries, leading to microvascular occlusion and therefore painful vaso-occlusive crisis [4]. Healthy RBCs typically live 90-120 days, but sickle cells only survive 10-20 days. Factors that cause this sickle cell crisis include infections, low oxygen tension, dehydration, acidosis, extreme physical exercise, physical or psychological stress, alcohol, pregnancy and cold weather [5].

Phytochemicals in plant extracts have therapeutic activities among which include antibacterial effect of Carica papaya root extract [6], antimicrobial effect of Zanthoxylum zanthoxyloides root extract [7], antioxidative effect of Carica papaya leaf extract [8], antisickling effect of Cajanus cajan seed extract [9]. They are used in traditional practice by traditional healers. However recent studies support the claims of traditional healers and suggest the possible correlation between the chemical composition of these plants and their uses in traditional medicine [10]. Aqueous and ethanolic extracts of several phytomedicines have been evaluated for significant In-vitro antisickling activity, some of them have been reported to have antisickling properties and others reversal of sickling activity [11].

Papaya leaf extract is derived from the leaves of the papaya tree commonly found in tropical areas. The nutrients in papaya leaf extract include the minerals, magnesium, potassium iron, most amino acids and vitamin A, C and B. The leaves of Carica papaya have been shown to contain many active components that can increase the total antioxidant power in blood and reduce lipid peroxidation level, such as papain, chymopapain, cystain, tocopherol, ascorbic acid, flavonoids, cyanogenic glucoside and glucosinolates [12].

The aim of this study is to evaluate the antisickling property and membrane stabilizing potential of Carica papaya leaf extract using in vitro antisickling tests and osmotic fragility test respectively.
2. MATERIALS AND METHODS

2.1 Location of Study

This study was carried out in Delta State University Clinic, Abraka. Located in local Government Area, Delta State.

2.2 Ethical Consideration

Ethical approval was received from the research ethical committee of faculty of Basic Medical Sciences, College of Health Sciences, Abraka, Delta State.

2.3 Sample Size

Fifty confirmed sickle cell patients aged between 12 and 45 years, who came for check-up and treatment in Delta State University Clinic, Abraka, Delta State. Both males and females were selected consecutively. The sample size was gotten as follows:

\[ n = \frac{Z^2 \cdot p \cdot q}{d^2} \]

where

- \( n \) = sample size
- \( p \) = prevalence
- \( q = (1-p) \)
- \( Z = 95\% \) confidence interval set at 1.96
- \( d = \) degree of accuracy – 0.05.
- \( n= (1.96)^2 \times 0.023 \times 0.977 / 0.0025 = 34.5 \)
  at prevalence of 2.3%.

2.4 Plant Materials

The leaves of *Carica papaya* was collected from Delta state University, Farm Site, Delta state, Nigeria. The plant was identified by Mr. F.O. Odili of the Department of Plant Science, Faculty of Agriculture, Delta State University, Anwai Delta State Nigeria. Voucher no FHI 106933 of plant leaves deposited in the herbarium of the forestry research institute of Nigeria, Ibadan.

2.5 Preparation of Papaya Leaf Extract

*Carica papaya* leaves was collected and dried at 400C in an oven. The dried leaves was grinded in a cross beaker mill equipped with a 1mm sieve, an aliquot (400 g) was homogenized in 100 ml of water and extracted by evaporation using electrical evaporator extraction apparatus (rotary evaporator), at a temperature of 45°C and pressure of 60 cm of water [13]. The extract was then stored in a refrigerator for later use.

2.6 Chemicals and Reagents

All the chemicals used were purchased from British Drug House (BDH) England. They include: Sodium metabisulphite, Formalin, NaHPO\(_4\), NaH\(_2\)PO\(_4\), NaOH, Liquid paraffin.

2.7 Study Design

In this experiment 50 samples of HbSS blood where used. The samples were divided into five groups, with ten (10) samples in each group. The samples in the different groups were treated with different concentration of the extract ranging from 2 mg/ml to 10 mg/ml.

- **Group 1** = The samples were treated with 2 mg/ml concentration of *Carica papaya* leaf extract.
- **Group 2** = The samples were treated with 4 mg/ml concentration of *Carica papaya* leaf extract.
- **Group 3** = The samples were treated with 6 mg/ml concentration of *Carica papaya* leaf extract.
- **Group 4** = The samples were treated with 8 mg/ml concentration of *Carica papaya* leaf extract.
- **Group 5** = The samples were treated with 10 mg/ml concentration of *Carica papaya* leaf extract.

For each sample three different tests were carried out;

1. Osmotic fragility test;
   - For osmotic fragility test, two test tubes were set up;
     - One control without *Carica papaya* leaf extract and
     - Experiment with *Carica papaya* leaf extract.

2. Inhibitory antisickling assay
   - For inhibitory anti-sickling assay, two test tubes were set up;
     - One control without *Carica papaya* leaf extract and
     - Experiment with *Carica papaya* leaf extract.
3. Reversal anti-sickling assay,

For reversal anti-sickling assay, two test tubes were set up;

- One control without Carica papaya leaf extract and
- Experiment with Carica papaya leaf extract.

### 2.8 Collection of Blood Sample

Fresh blood samples were collected from confirmed sickle cell patients with their full consent at the Delta State University health centre, Abraka, Delta State. 5 ml each of fresh blood sample was drawn from the vein by venopuncture into EDTA (ethylene diaminotetraacetic acid) bottles from sickle cell anemia patients in steady state, both males and female. The blood was mixed carefully and was used within 72 hours of collection.

### 2.9 Inhibitory Antisickling Assay

To test for the anti-sickling effect of Carica papaya leaf extract, a previously described method was used [14]. HbSS whole blood (0.2 ml) was pipetted into test tubes then 0.2 ml Phosphate Buffered Saline Solution (pH 8.0) and 0.2 ml of Carica papaya leaf extracts were added. The mixture was overlaid with liquid paraffin (1ml) and incubated in a thermostated water bath at 37°C for 4 hours. Then freshly prepared 2% sodium metabisulphite solution (0.6 ml) was carefully added under the liquid paraffin to the mixture. The mixture was thoroughly mixed and incubated further for another 90 minutes at 37°C in a water bath. The experiment was setup in duplicates with a negative control where 0.2 ml phosphate buffered solution was used in place of the extract. After 90 minutes of incubation, liquid paraffin was carefully removed using a Pasteur pipette and 5% buffered formalin solution (3 ml) was added at the end of the 4 hour incubation. The solution was thoroughly mixed to ensure proper fixation and was kept at ambient temperature until ready for counting. Prepared slide was viewed under microscope. The percent of normal RBCs and sickled cells were counted to monitor the level of sickling reversal of the plant treatment.

### 2.10 Reversal Antisickling Assay

To determine the sickle cell reversal effect of Carica papaya leaf extract, reversal antisickling assay as previously described [15,16] was used. HbSS blood (0.2 ml) was pipetted into a test tube, and 0.2 ml Phosphate Buffered Solution (PBM, 0.005M, PH 8.0) was added. The mixture was covered with liquid paraffin (1.0 ml). 2% sodium metabisulphite solution (0.6 ml) was introduced into the mixture under the liquid paraffin using a syringe and needle. The mixture was mixed gently before incubating at 37°C in a thermostated water bath for 90 minutes. At the end of incubation period, the aqueous extract (0.2 ml) of Carica papaya leave was added under the liquid paraffin carefully and incubated for another 4 hours. The experiment was setup in duplicates with a negative control where 0.2 ml phosphate buffered solution was used in place of the extract. The liquid paraffin layer was carefully removed using a Pasteur pipette and 5% buffered formalin solution (3 ml) was added at the end of the 4 hour incubation. The solution was thoroughly mixed to ensure proper fixation and was kept at ambient temperature until ready for counting. Prepared slide was viewed under microscope. The percent of normal RBCs and sickled cells were counted to monitor the level of sickling reversal of the plant treatment.

### 2.11 Osmotic Fragility Test

The osmotic fragility of erythrocytes measures the membrane stability effect of the extract in osmotic stress/ hypotonic lysis after 30 minutes incubation. The protocol by [17,11] was used for the analysis, with some modifications. To 10ml reaction vessels containing 4ml of buffered saline PH 7.4, 1 ml of a range of concentrations of extract (2mg/ml to 10 mg/ml) and 0.05 ml HbSS blood was added. The mixture was left to incubate at room temperature for 30 minutes and then centrifuged at 2000 rpm for 15 minutes. The experiment was setup in duplicates with a control where Carica papaya leaf extract was not added to the mixture. Then test supernatant was collected and read at 540 nm against blank (0.85% buffered saline concentration).

### 2.12 Statistical Analysis

The data was expressed as mean ± standard deviation (S.D) and analysed using Students T-test and One Way Analysis of Variance (ANOVA) to compare the control and experimental groups at p<0.05 level of significance, using statistical package for Social Science (SPSS) software package version 20.

### 3. RESULTS

Table 1 shows the antisickling effects of Carica papaya leaf extract on HbSS red blood cells. The result showed a significant reduction in percentage sickled red blood cells of experiment...
groups when compared to their control groups. Group 1 had percentage sickling of 37.00±21.76 compared to control which had 57.50±21.63, group 2 had percentage sickling of 38.00±13.98 compared to control which had 66.00±17.76, and group 3 had percentage sickling of 31.50±13.55 compared to control which had 69.00±16.47 while group 4 had 31.50±14.92 compared to 73.00±14.94 of control and group 5 had 29.00±15.24 compared to 81.00±18.07 of control. Thus the antisickling activity was dose dependent.

Table 2 shows the effects of Carica papaya on In-vitro reversal of sickling Activity on HbSS red blood cells. The result showed significant reduction in percentage sickled red blood cells in all experimental groups compared to their control groups. Group 1 had percentage sickling of (33.20±14.38) compared to control which had (60.00±11.06). Group 2 had percentage sickling of (37.00±13.78) compared to control that had (67.00±13.38). Group 3 had percentage sickling of (31.80±14.23) compared to control that had (68.50±11.80). Group 4 had percentage sickling of (25.00±15.81) compared to control which had (67.80±18.73). Group 5 had percentage sickling of (17.20±11.76) compared to control which had (83.80±11.45). This effect was also dose dependent.

In the management of tropical diseases and genetic disorders like sickle cell anemia, research in phytotherapy is a current trend, with the view of finding cheaper, readily available and alternative medicines. Some tropical plants have been found to have antisickling properties and are employed in the management of sickle cell disease [19].

### Table 1. Anti-sickling effect of Carica papaya on HbSS red blood cells

| Experiment | Control | t-stat | t critical | P value | Remarks |
|------------|---------|--------|------------|---------|---------|
| Group 1    | 37.00±21.76 | 57.50±21.63 | 4.904 | 1.833 | P<0.05 | significant |
| Group 2    | 38.00±13.98 | 66.00±17.76 | 5.782 | 1.833 | P<0.05 | significant |
| Group 3    | 31.50±13.55 | 69.00±16.47 | 7.319 | 1.833 | P<0.05 | significant |
| Group 4    | 31.50±14.92 | 73.00±14.94 | 11.857 | 1.833 | P<0.05 | significant |
| Group 5    | 29.00±15.24 | 81.00±18.07 | 10.614 | 1.833 | P<0.05 | significant |

Values are expressed as mean ± Standard Deviation (S.D), n=10. P<0.05: Significant as determined by student's T-test.

### Table 2. Reversal of sickling effect of Carica papaya on HbSS red blood cells

| Experiment | Control | t-stat | t critical | P value | Remarks |
|------------|---------|--------|------------|---------|---------|
| Group 1    | 33.20±14.38 | 60.00±11.06 | 4.431 | 1.833 | P<0.05 | Significant |
| Group 2    | 37.00±13.78 | 67.00±13.38 | 6.285 | 1.833 | P<0.05 | Significant |
| Group 3    | 31.80±14.23 | 68.50±11.80 | 8.419 | 1.833 | P<0.05 | Significant |
| Group 4    | 25.00±15.81 | 67.80±18.73 | 9.112 | 1.833 | P<0.05 | Significant |
| Group 5    | 17.20±11.76 | 65.50±16.24 | 6.697 | 1.833 | P<0.05 | Significant |

Values are expressed as mean ± Standard Deviation (S.D), n=10. P<0.05: Significant as determined by student's T-test.

### 4. DISCUSSION

Sickle cell disease is a genetic disease that affects hemoglobin causing it to assume a sickled shape that cannot effectively carry oxygen to tissue and organs. This abnormality results in crises and may manifest as anemia, pain, fever, jaundice, leg ulcer [18]. It has been difficult to find an efficacious cure for this disease because of its genetic origin, even though it can be managed by using some medications such as hydroxyurea [2].

In the management of tropical diseases and genetic disorders like sickle cell anemia, research in phytotherapy is a current trend, with the view of finding cheaper, readily available and alternative medicines. Some tropical plants have been found to have antisickling properties and are employed in the management of sickle cell disease [19].
This study which investigated the anti-sickling effect and membrane stabilizing activities of aqueous Carica papaya leaf extract, showed a high level of anti-sickling effect (Table 1), reversal of sickling effect (Table 2) and high membrane stabilizing activity (Fig. 1) on HbSS red blood cells. There was significant (p<0.05) reduction in the mean percentage of the sickled red blood cells in experimental groups compared to the mean percentage of sickled red blood cells in control groups in the antisickling and reversal of sickling experiments. There was significant decrease in absorbance of experimental groups compared to control groups at (p<0.05) in membrane stabilizing experiments. These effects were dose dependent.

This observation may have occurred due to the high level of phytochemicals inherent in plants as previous phytochemical studies of Carica papaya indicated the presence of high concentrations of alkaloids and antioxidant such as papain, ascorbic acid, flavonoids, chymopapain, cyanogentic glucosides, cystatin and glucosinolates which were found to increase the total antioxidant power in the blood and reduce the oxidative damage [20]. However the exact phytochemical responsible for this activity yet to be determined.

The observations in this study is in agreement with previous study; Anti-sickling agent in an extract of unripe pawpaw (Carica papaya) [21].

Anti-sickling agents have been reported to prolong delay time of Hb polymerization as part of the mechanisms for its antisickling action [21].

However in this study, Carica papaya leaf extract was not found to prolong the delay time but inhibited HbSS polymerization indicating that the extract may apply a target hit on HbSS polymerization in attenuating HbSS red blood cell sickling, unlike other reported compounds whose antisickling actions are premised on the interaction with HbSS molecules [22,23]. There is also an indication that the effect of the extract is probably at the cell membrane level and not direct interaction with HbSS molecules. As the erythrocyte membrane stability effect of the Carica papaya leaf extract analyzed using osmotic fragility test, showed appreciable membrane protective effects of the extract and its inhibitory action on the hemolysis of RBCs.

The toxicity profile of the plant as assessed by histological and biochemical analyses did not reveal any substantial toxicity of the plant [11]. Other tables and figure are presented in the appendix.

5. CONCLUSION

This study has conclusively shown that Carica papaya leaf extract inhibits sickling of HbSS red blood cells and also reverses sickling of already sickled HbSS red blood cells. It also showed that Carica papaya leaf extract has membrane stabilizing effect on HbSS red blood cells. Thus this result indicates the feasibility of Carica papaya leaf extract as an attractive potential candidate for SCD therapy and strongly collaborates the ethnomedical usage of the plant which lies in its availability and affordability.
COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Table A. Percentage sickled and non-sickled cells after sickling test

| Experiment | Control |
|------------|---------|
| % sickled  | % normal| % sickled  | % normal  |
| Group 1(2mg/ml) | 37.00±21.76 | 63.00±21.76 | 57.50±21.63 | 42.50±21.64 |
| Group 2(4mg/ml) | 38.00±13.98 | 62.00±13.98 | 66.00±17.76 | 34.00±17.76 |
| Group 3(6mg/ml) | 31.50±13.55 | 68.50±13.55 | 69.00±16.47 | 31.00±16.47 |
| Group 4(8mg/ml) | 31.50±14.92 | 68.50±14.92 | 73.00±14.94 | 27.00±14.94 |
| Group 5(10mg/ml) | 29.00±15.24 | 71.00±15.24 | 81.00±18.07 | 19.00±18.07 |

Values are expressed as mean ± Standard Deviation (S.D), n=10

Table B. Presentation of percentage sickled and non-sickled cells in control and experiment groups after reversal of sickling procedure

| Experiment | Control |
|------------|---------|
| % sickled  | % normal| % sickled  | % normal  |
| Group 1(2mg/ml) | 33.20±14.38 | 66.80±14.38 | 60.00±11.06 | 40.00±11.06 |
| Group 2(4mg/ml) | 37.00±13.78 | 63.00±13.78 | 67.00±13.38 | 33.00±13.38 |
| Group 3(6mg/ml) | 31.80±14.23 | 68.30±14.26 | 68.50±11.80 | 31.50±11.80 |
| Group 4(8mg/ml) | 25.00±15.81 | 75.00±15.81 | 67.80±18.73 | 32.20±18.73 |
| Group 5(10mg/ml) | 17.20±11.76 | 83.80±11.45 | 65.50±16.24 | 34.00±15.78 |

Values are expressed as mean ± Standard Deviation (S.D), n=10

Table C. Osmotic fragility showing the membrane stabilizing effect of Carica papaya on HbSS red blood cell

| Experiment | Control |
|------------|---------|
| t stat     | t critical | P <value | Remark |
| Group 1(2mg/ml) | 0.36±0.07 | 0.70±0.09 | 19.207 | 1.833 | 0.05 | Significant |
| Group 2(4mg/ml) | 0.45±0.085 | 0.81±0.09 | 23.232 | 1.833 | 0.05 | Significant |
| Group 3(6mg/ml) | 0.46±0.10 | 0.86±12 | 20.281 | 1.833 | 0.05 | Significant |
| Group 4(8mg/ml) | 0.43±0.09 | 0.85±16 | 13.161 | 1.833 | 0.05 | Significant |
| Group 5(10mg/ml) | 0.40±0.17 | 0.89±0.29 | 6.020 | 1.833 | 0.05 | Significant |

Values are expressed as mean ± Standard Deviation (S.D), n=10,
P<0.05: Significant as determined by student's T-test

Fig. 1. Antisickling effect of Carica papaya leaf extract on HbSS red blood cells
Fig. 2. Showing the reversal of sickling effect of extract

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