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Invitro Membrane Stabilizing Potentials Of Fractionates Of Ethanolic Extract of *Carica Papaya* Leaf

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ABSTRACT

Invitro membrane-stabilizing potentials of fractionates of ethanolic extract of *Carica Papaya* leaf was investigated in this study. The soxhlet extraction method was used to *extract the plant*, fractionated with 6 different solvents to give 6 different fractions (hexane, ethyl acetate, chloroform, butanol, methanol benzene). Hbss red blood cells samples were obtained from non-crises state sickle cell patients from Eku Baptist hospital Abraka Delta State, Nigeria. These tests involved the use of positive (p-hydroxy benzoic acid 5ug/ml) and negative controls (normal saline) for membrane stability experiments. Hbss blood was treated with 2mg/ml to 10mg/ml in seven groups with leaf fractionates. Data was analyzed using ANOVA test. The results shows that osmotic fragility was reduced by the introduction of the leaf fractionate, with the highest rate of reduction noticed in the hexane 1 fractionate. PHBA reversal rate and osmotic fragility effect was normal at low doses, but as concentration increases, reversal rate and percentage reduction of sickling decreases. It was concluded that *Carica papaya* leaf extract fractions, just as its crude extracts, have as much osmotic fragility activities, and this is dose-dependent and has no negative effect on tested blood samples as compared with the treatments with PHBA.

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INTRODUCTION

Sickle cell disease (SCD) is a potentially devastating condition caused by an autosomal recessive inherited hemoglobinopathy, which results in the hallmark clinical sequel 21 of vasoocclusive phenomena and hemolysis. Sickle cell disease was first described more than a hundred years ago (Pauling and Itano, 1949). Hemoglobin S (HbS), the hemoglobin that is produced as a result of this defect, is a hemoglobin tetramer (alpha2/beta S2) that is poorly soluble and polymerizes when deoxygenated (Guyton and Hall, 2006). Globally, the incidence of sickle cell disease exceeds most other serious genetic disorders, including hemophilia and cystic fibrosis, by Platt et al. Sickle cell anemia is a chronic, lifelong disease (Emojevwe and Igweh, 2012). (Angastiniotis and Modell, 1998) posited that SCD is prevalent worldwide but occurs most frequently in Africans and less so in the Mediterranean,

Latino, East Indian, and Arab descent.

Red blood cells are biconcave discs with an average diameter of 7.8 micrometers. They are 1 micrometer thick or less in the middle and 2.5 micrometers thick at the thickest tip. The typical red blood cell volume is 90 to 95 cubic micrometers (Connes *et al.*, 2008). When red blood cells are transferred from the bone marrow into the circulatory system, they usually circulate for about 120 days before being destroyed (Guyton and Hall, 2006). Sickled erythrocytes tend to block capillaries in vivo, causing stasis and thereby starve organs of both nutrients and oxygen, leading to organ damage. In vitro studies have portrayed that some osmotic fragility agents, especially those of plant origin, affect the kinetics of hemoglobin (Hb) polymerization and inhibit the duration for red blood cell sickling (Iyamu *et al.*, 2002). The pathophysiology of SCD is centered on the decrease of RBC flexibility (Lazarus and Schmaier, 2011; Emojevwe and Igweh, 2012).

Carica papaya is an evergreen shrub that grows best in full sun to light shade (Sugiharto, 2020). The *papaya* plant has been described in so many ways, which acknowledge the functional and structural and complexity of this giant tropical plant (Carvalho and Renner, 2013; Jiménez *et al.*, 2014). *C. papaya* has always been a fascinating plant to lots of researchers. It is a power house of nutrients and is available throughout the year. *C. papaya* has 18 somatic chromosome numbers, and it is the only species of the genus *Caricaceae,* a family represented in the Neotropics, which includes six genre with a minimum of 35 species (Jiménez *et al.*, 2014). The leaves of papaya had been proved to contain different active components like; enzymes, alkaloids, flavonoids, electrolytes and minerals, phenolic compounds, glycoside, glucoside, carotenoids, vitamins, amino acids, among others (Krishna *et al.*, 2008).

A multi-phased strategy integrating botanical, biological, phytochemical, and molecular methods is required for drug development from medicinal plants. Medicinal plant drug development continues to yield novel and significant leads against a wide range of pharmacological targets, including malaria, pain, cancer, HIV/AIDS, Alzheimer's disease, and typhoid (Haider, 2013). Unfortunately, only about 7% of patients with the disease meets the criteria for transplantation. Birth prevalence in Nigeria, Its treatment has proven difficult due to its genetic origin. This study will provide information about the invitro membrane-stabilizing potential of ethanolic extract of *Carica papaya* leaf on HbSS RBCs using osmotic fragility test, with a view of developing new means of prevention of crisis in SCD patients.

MATERIALS AND METHODS

Participants

All the sickle cell disease patients who visited Sickle Cell Clinic at Eku Baptist Government Hospital, Eku, Delta State, male and female within the age of 14-45 years form the study population. In this experiment, 50 samples of HbSS blood were used. The samples were divided into five groups, with seven (7) samples in each group. The samples in the different groups was treated with different concentration of the extract fractionate ranging from 2mg/ml to 10mg/ml.

Sample Size and Sampling

The sample size of 50 (males and females) confirmed sickle cell patients in crisis Free State who came for checkups and treatment at the Eku Baptist Government Hospital Eku, Delta State. The sample size was gotten adopting the formula below:

$$n = Z^2 Pq/d^2$$

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 x 0.023 x 0.977 / 0.0025 = 34.5

at prevalence of 2.3%

The consecutive sampling technique was used. Anyone who met the selection criteria was recruited as the number of people with sickle cell anemia.

Materials and Chemicals

The following apparatus and instruments was used for this project: Microscope, Centrifuge (model 8000), VIS 722N Spectrophotometer, meter 200 Electronic weighing balance, Incubator, methanol, Water bath, Oven, Distiller, micro pipette, slides and test tubes, soxhlet apparatus, retort stand, clamp, measuring cylinder, separating funnel. All chemicals used was purchased from the British Drug House (BDH) England by Sea gold scientific store; They include Sodium metabisulphite, Formalin, $NaHPO_4$, NaH_2PO_4, NaOH, Liquid paraffin, P-hydroxybenzoic acid,), chloroform, hexane, ethanol, methanol, HCL, H_2SO_4, butanol, ethylacetate.

Preparation of Papaya Leaf Extract

Carica papaya leaf was collected and dried at room temperature. The dried leaves were grinded in a cross beaker mill, equipped with a 1mm sieve.

An aliquot (400g) was homogenized in ethanol (100ml) and extracted by evaporation, using evaporator extraction apparatus (Soxhlet extraction) at 45^{o}C and 60ml of ethanol. The extracts was stored in a refrigerator for later use.

Preparation of papaya leaf fractionate

Crude ethanolic extract of *Carica papaya* leaf was fractionated, using a serial liquid-liquid separation method as described by Masfufatun et al.. 200ml of crude extract was measured using a retort stand and a clamp. 200ml of benzene will be added to it, shaken properly and allowed to lyse for 30 minutes. The mixture separated into two layers, a layer containing benzene soluble constituent of *C. papaya* leaf extract, which was collected into a beaker, the other layer containing non-benzene soluble residue. The resultant residue will be left to air dry to remove traces of benzene. After drying, the 200ml of the residue will be measured into a separating funnel and 200ml of chloroform was added to it and shaken properly and left to stand for 30 minutes as above. The mixture was separated into two layers (chloroform soluble constituent and non-chloroform soluble residue). The non-chloroform soluble phase will be collected, allow to air dry and then put into another separating funnel and further fractionated using ethyl acetate, butanol, ethanol, hexane.

Preparation of Different Concentrations of fractionate

Five different concentrations of extract and fractionate (7) was prepared. 2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml.

1. For 2mg/ml, 2mg of extract and fractionate was weighed using an analytical weighing machine and was dissolved in 1ml of distilled water.
2. For 4mgml, 4mg of extract and fractionate was weighed using an analytical weighing machine and was dissolved in 1ml of distilled water.
3. For 6mg/ml, 6mg of extract and fractionate was weighed using an analytical weighing machine and was dissolved in 1ml of distilled water.
4. For 8mg/ml, 8mg of extract and fractionate was weighed using an analytical weighing machine and was dissolved in 1ml of distilled water and
5. For 10mg/ml, 10mg of extract and fractionate was weighed using an analytical weighing machine and was dissolved in 1ml of distilled water.

Selection Criteria

The criteria for subject selection include health status and subjects willingness to partake in the study; Sickle cell disease patients who were apparently healthy were recruited, Sickle cell disease patients who were not on any herbal medications for sickle cell disease were recruited. The exclusive criteria includes; sickle cell patients suffering from crises and sickle cell patients with known comorbidity.

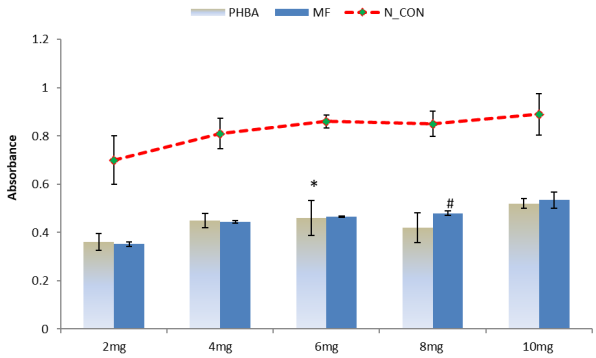

Figure 1: Comparative illustrations of osmotic fragility test in Methane fractionate

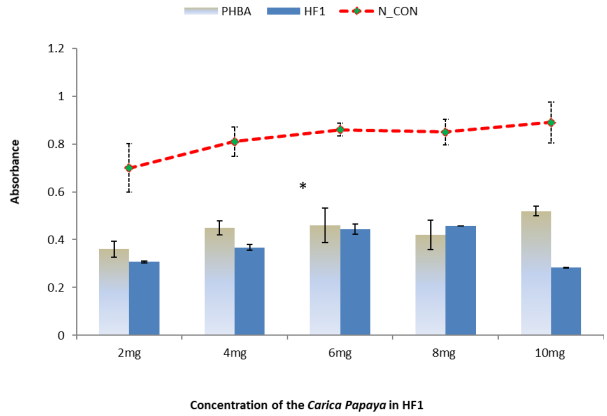

Figure 2: Comparative illustration of Osmotic Fragility test of *C. papaya* Hexane fractionate

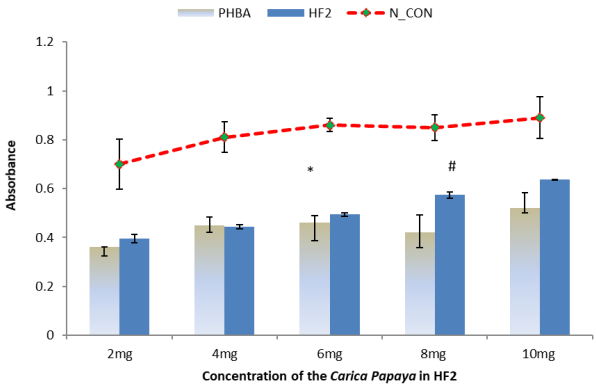

Figure 3: Comparative illustration of Osmotic Fragility test of *C. papaya.* Hexane fractionate 2

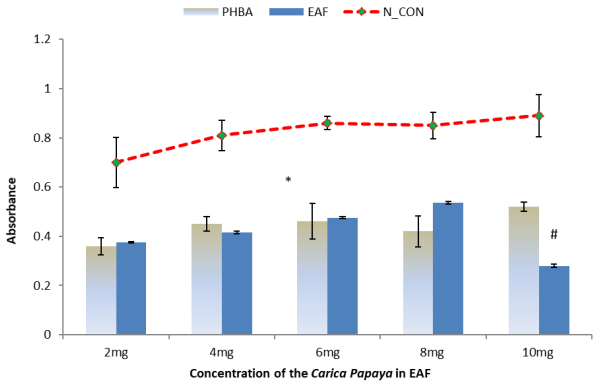

Figure 4: Comparative illustration of Osmotic Fragility test of *C. papaya.* Ethyl acetate fractionate

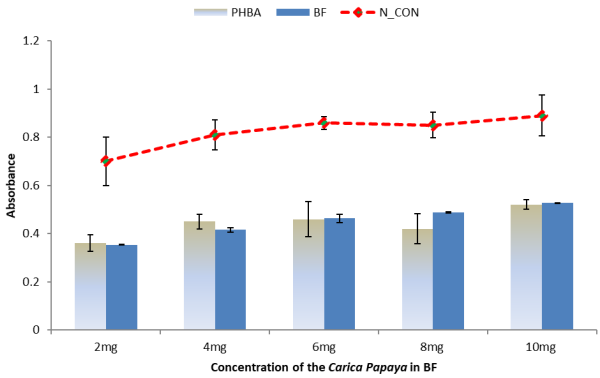

Figure 5: Comparative illustration of Osmotic Fragility test of *C. papaya* Butanol fractionate

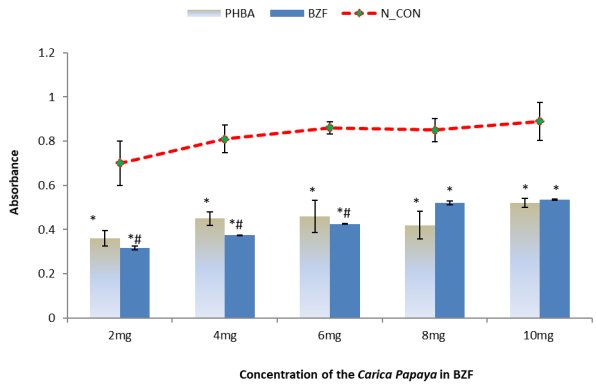

Figure 6: Comparative illustration of Osmotic Fragility test of *C. papaya* Benzene fractionate

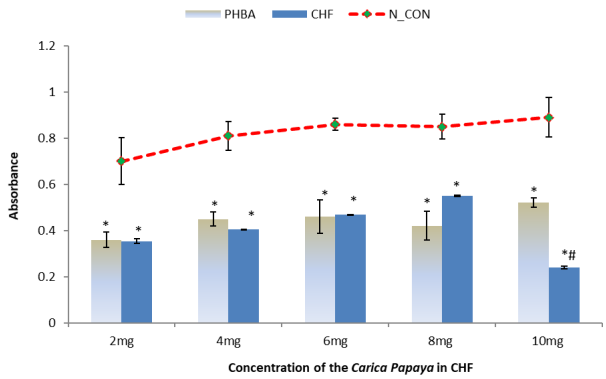

Figure 7: Comparative illustration of Osmotic Fragility test of *C. papaya.* Chloroform fractionate

Ethical Consideration

A letter of approval was sought and received from the Hospital Management Board Eku Baptist Government Hospital. Subjects were properly and adequately informed, and their consents gotten. Ethical approval was also collected from the Ethics and Grant Committee of the Faculty of Basic Medical Sciences, Delta State University, before the commencement of the study.

Sample Collection

A fresh blood sample was collected from confirmed sickle cell patients, with their full informed consent at the Eku Baptist Government Hospital, Abraka, Delta State. 5ml each of fresh blood sample was drawn from the vein by vein puncture into EDTA (ethylene di amino tetra acetic acid) bottles from sickle cell anemia patients in steady-state, both males and females. The blood was mixed carefully and used within 72 hours of collection.

Biochemical Examination of Membrane Stabilizing Activity

The membrane-stabilizing assay was carried out using the procedures of Faladeet al., with some modifications. The assay mixture consisted of 2ml of 0.25% (w/v) sodium chloride, 1.0 ml of 0.15M sodium phosphate buffer (pH 7.4), concentration of plant extracts (2, 4, 6, 8, 10mg/ml) and 0.5ml of (2% v/v) erythrocyte suspension. The control was prepared as above, first control with normal saline and second control with PHBA; the experimental group was set as above with different concentrations of fractions. The reaction mixtures were incubated at 560C for 30 minutes, cooled under running water and then centrifuged at 3913 x g. The principle adopted the spectrophotometric measurement (read at 560nm) of the amount of hemoglobin released by sickled erythrocytes, which is depen-

dent on the extent of stabilization of sickled RBC membrane exerted by fractions and the test extract. The first control, which was the negative control, was without extract, it consists of 2mg of normal saline solution to replace the extract. The second control, which is the positive control, was p-hydroxyl benzoic acid (PHBA).

Statistical Analysis

The data was expressed as mean ± standard Error. It was analyzed using One Way Analyses of Variance and (ANOVA) to compare the control and experimental groups, at p<0.05 level of significance, followed by *Tukey's post hoc* test for multiple comparisons.

RESULTS

Figure 1 shows the effect of *carica papaya* on in-vitro osmotic fragility. The result showed a significantly reduced absorbance when PHBA and fractionate extract was compared with the negative control in all concentration. At 8mg, MF showed the most significant reduction of absorbance when compared with PHBA. In Figure 1 Values are expressed as Mean±SEM (n=5) as determined by One-Way ANOVA followed by Tukeys'post hoc test *, # statistically significant different at p<0.05 when compared with N.CON and PHBA, respectively. **Key:** MF-methanol fractionate, N.CON-Negative control. PHBA-Positive control

Figure 2 shows the effect of *Carica papaya* on in-vitro osmotic fragility. The result showed a significantly reduced absorbance when PHBA and fractionate extract was compared with the negative control in all concentration. HF1 at 2mg, 4mg, 6mg, 10mg showed the most significant reduction of absorbance when compared with PHBA. In Figure 2 Values are expressed as Mean±SEM (n=5) as determined by One-Way ANOVA followed by Tukeys'post hoc test *, # statistically significant different at p<0.05 when compared with N.CON and PHBA, respectively. **Key:** HF1- hexane fractionate 1

Figure 3 shows the effect of *Carica papaya* on in-vitro osmotic fragility. The result showed a significant reduced absorbance when PHBA and fractionate extract was compared with the negative control in all concentration. HF2 at 4mg showed a slight significant higher in absorbance when compared with PHBA. In Figure 3*Values are expressed as Mean±SEM (n=5) as determined by One-Way ANOVA followed by Tukeys'post hoc test *, # statistically significant different at p<0.05 when compared with N.CON and PHBA respectively.* **Key:** HF1- hexane fractionate 2

Figure 4 shows the effect of *Carica papaya* on *in-vitro* osmotic fragility. The result showed a significantly reduced absorbance when PHBA and fractionate extract were compared with the negative control across all concentrations adopted in this study. EAF showed a significant reduction of absorbance when compared with the PHBA at 10 mg. In Figure 4 Values are expressed as Mean±SEM (n=5) as determined by One-Way ANOVA followed by Tukeys'post hoc test *, # statistically significant different at p<0.05 when compared with N.CON and PHBA respectively. **Key:** EAF- ethyl acetate fractionate

Figure 5 shows the effect of *Carica papaya* on in-vitro osmotic fragility. The result showed a significant reduced absorbance when PHBA and fractionate extract was compared with the negative control in all concentration. BF at 4mg showed the most significant reduction of absorbance when compared with the PHBA. In Figure 5 Values are expressed as Mean±SEM (n=5) as determined by One-Way ANOVA followed by Tukeys'post hoc test *, # statistically significant different at p<0.05 when compared with N.CON and PHBA respectively. **Key:** BF-butanol fractionate

Figure 6 shows the effect of *Carica papaya* on in-vitro osmotic fragility. The result showed a significantly reduced absorbance when PHBA and fractionate extract was compared with the negative control in all concentration. BZF at 2mg, 4mg 6mg showed a significant reduction of absorbance when compared with the PHBA. In Figure 6 Values are expressed as Mean±SEM (n=5) as determined by One-Way ANOVA followed by Tukeys'post hoc test *, # statistically significant different at p<0.05 when compared with N.CON and PHBA respectively. **Key:** BZF- Benzene fractionate

Figure 7 shows the effect of *Carica papaya* on in-vitro osmotic fragility. The result showed a significant reduced absorbance when PHBA and fractionate extract was compared with the negative control in all concentration. CHF at 4 mg and 10 mg showed a significant reduction in absorbance when compared with the PHBA. In Figure 7Values are expressed as Mean±SEM (n=5) as determined by One-Way ANOVA followed by Tukeys'post hoc test *, # statistically significant different at p<0.05 when compared with N.CON and PHBA respectively. **Key:** CHF- Chloroform

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 of finding cheaper alternative medicine that the wide population can have immediate access to. Once the membrane integrity in compromised, the cell becomes so fragile, making it more sickled and unable to squeeze through the narrow capillaries. This function of the RBC membrane is normally determined by measuring the absorbance of the membrane. Recent studies shows that *C. papaya* leaf crude extract has the effect of extract fractionate on membrane stability in HBss blood. This is in accordance with previous studies of (Naiho *et al.*, 2015), which reported the membrane-stabilizing effect of *Carica papaya* leaf extract after investigation. HF 1 showed a statistically significant result in membrane stability in concentrations 2mg, 4mg, 8mg. Membrane integrity is important for the normal functioning of the Red cell. According to (Orbach *et al.*, 2017), the absorbance is negatively correlated to membrane stability. This higher the absorbance, the lesser the integrity of the membrane and the more fragile the RBC becomes.

The result showed a significant reduced absorbance when PHBA and fractionate extract was compared with the N. control in all concentration. MF at 8mg showed the most significant reduction of absorbance when compared with the crude extract. Results obtained showed a significant reduced absorbance when PHBA and fractionate extract was compared with the normal control in all concentration. Similarly, the result showed a significant reduced absorbance when PHBA and fractionate extract was compared with the normal control in all concentration. HF2 at 4mg showed a slight significant reduction of absorbance when compared with the crude extract, and the crude extract showed a more significant reduction. This result is in accordance with a previous study by (Imaga and Olusegun, 2010), which reported the analyses of the potency of *Carica papaya* dried leaf extract and fractions in membrane stability of sickled of HbSS cells.

Results obtained from the present study showed a significant reduced absorbance when PHBA and fractionate extract was compared with normal control in all concentration. In addition, there was a significant reduced absorbance when PHBA and fractionate extract was compared with normal control in all concentration. The observed reduction in the absorbance when PHBA and fractionate extract was significant when compared with the normal control in all concentrations. BZF at 2mg, 4mg 6mg showed the most significant reduction of absorbance when compared with the crude extract. Finally, the data from this study has shown that there was significant reduction absorbance when PHBA and fractionate extract was compared with normal control in all concentration. According to (Iyamu *et al.*, 2002), *C. papaya* leaf extract and fractions inhibited haemoglobin polymerization in the RBC suspension and thus inhibited the time course for sickling of HbSS cells.

CONCLUSIONS

The study established that the invitro membrane stabilizing activities of fractionates of *Carica papaya* leaf extract increased with a concentration in contrast to PHBA that had a decline in membrane stabilizing activities as concentration increases. Hence, the study has shown that fractionate crude extracts of *Carica papaya* possesses in-vitro membrane stability potentials depending on the dosage. The result further indicates the possibility of *Carica papaya* as a potential phytomedicine for SCD therapy.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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