

The In-Vitro Effect of Aqueous Leaf Extract of *Basella Alba* L. On Osmotic Fragility of Red Blood Cells in Hbss Subjects

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Abstract

The aqueous leaf extracts of *Basella alba* were investigated in the present study for their in-vitro effect on red blood cell fragility in clinically confirmed HbSS subjects. A total of 12 human subjects, six (6) male and six (6) female with HbSS were used for this study. In-vitro osmotic fragility was determined spectrophotometrically by obtaining optical density at wavelength of 540nm. 5-6 ml of blood was obtained from each subject and analyzed with the use of 15 centrifuge tubes, divided into 5 per group. The first group served as the control (without *Basella alba*), the second group contained low dose (0.2mg/ml) of *Basella alba* and the third group contained high dose (0.4mg/ml) of *Basella alba* in varying saline percentages of 0.0%, 0.3%, 0.6%, 0.9% and 1.2%. Hemolysis occurred at all saline percentages; however, low dose and high dose of aqueous extract of *Basella alba* reduced the percentage of hemolysis in all subjects, especially in the males. This suggests that there might be a possible synergistic activity between *Basella alba* and androgens, by which *Basella alba* reduces hemolysis of red blood cells, combined with its anti-anemic effect, thereby, maintaining the high red blood cell count seen in males. Thus, it is advised that *Basella alba* should be consumed regularly by sicklers, in order to help reduce the occurrence of hemolytic anemia.

Keywords: *Basella alba*, Osmotic fragility, Red blood cell, Sickle cell, Hemoglobin SS

Introduction

When red blood cells are placed in hypotonic solution in which osmolarity is diminished the gain in red blood cell water is both, instant and quantitative. This phenomenon is put into practical use in the red blood cell osmotic fragility test, which determines the release of hemoglobin from red blood cells in hypotonic sodium chloride (NaCl) solution. Therefore, osmotic fragility index is a measure of the resistance of red blood cells to lysis by osmotic stress (Oyewale and Ajibade, 1990) [20]. The test is generally useful to ascertain the level of stability and functionality of plasma membrane (Krogmeier *et al.*, 1993) [12] erythrocyte Mean Cell Volume (MCV), Surface Area-to-Volume Ratio (SAVR) and diagnosis of hereditary spherocytosis (Alderich *et al.*, 2006; Kumar, 2002) [1, 13] as well as diagnosis of thalassemia. Both of these genetic conditions (i.e. hereditary spherocytosis and thalassemia) can cause hemolytic anemia.

There are many types of hemolytic anemia, some of which are inherited and others acquired. An inherited hemolytic anemia, like Sickle cell anemia (SCA) is highly frequent in sub-Saharan Africa, the Middle East and Mediterranean areas, the Indian subcontinent, the Caribbean, and South America (Uzoegwu and Onwurah, 2003) [24]. The sickle erythrocyte hemoglobin (HbSS) disorder is caused by a point mutation affecting the coding sequence of the β -globin gene, causing a substitution of glutamic acid by valine at the sixth position of β -globins (Kutlar, 2005) [14]. This amino acid substitution leads to a drastic reduction in the solubility (gelation) of deoxy-HbSS molecules. Under low oxygen tension, deoxy-HbS molecules polymerize, causing the formation of rigid and sickled erythrocytes. The deformity of the sickled erythrocyte results in

their shortened survival since they become vulnerable to lysis as they penetrate the interstices of the splenic sinusoids and hence severe hemolytic anemia ensues with hemoglobin values ranging from 6 to 10 g/L (Martins, 1981; Karayakin, 1979) [16, 11]. The homozygous state of Sickle Cell Anemia (SCA) is associated with complications and a reduced life expectancy (Kutlar, 2005; Frenette and Atweh, 2007). [14, 8].

Some medicinal plant extracts have been demonstrated by *in vitro* investigations to reduce polymerization of HbSS molecules (Chikezie, 2006) [5] and have been established to serve as potential chemotherapeutic preparations for alleviation and management of SCA (Okpuzor *et al.*, 2008) [18]. For these agents to exert therapeutic benefit, they come in direct contact and interact with membrane architectural components and cellular processes required for erythrocyte functional and structural integrity.

Basella alba (Family: Basellaceae) known as Indian spinach is suspected to be one of the promising medicinal plants that may alleviate the plight of Sickle Cell Anemic patients owing to its involvement in the treatment of anemia and maintenance of good health (Bamidele *et al.*, 2010) [3]. It is high in vitamin A, vitamin C, vitamin B9 (folic acid), calcium, magnesium and several vital anti-oxidants. It is low in calories by volume and high in protein per calorie (Duke and Ayensu, 1985) [6]. Its leaves are used for the treatment of hypertension by Nigerians in Lagos (Olowokudejo *et al.*, 2008) [19], and malaria in cameroonian folk medicine (Vincent *et al.*, 2008) [26]. A literature survey revealed that it has antifungal (Premakumari *et al.*, 2010) [22], anticonvulsant, analgesic, anti-inflammatory (Kachchhava *et al.*, 2006) [10] and androgenic activities (Moundipa *et al.*, 2005) [17]. In the light of its uses, the present

research work was aimed to scientifically evaluate the in-vitro effect of aqueous extract of *Basella alba* leaves on red blood cell osmotic fragility in subjects with HbSS genotype.

Materials and Methods

Selection of Subjects

The medical records of subjects with HbSS genotype were obtained from Bowen University Medical Centre, Iwo, Osun State. Only those who have given oral consent were admitted into the study. The participants comprised of students with clinically confirmed HbSS erythrocyte genotype. A total of twelve (12) human subjects, six (6) male and six (6) female subjects were used for this study. All the Subjects were from Bowen University, Iwo, Osun State, Nigeria. The unit of ethical approval committee, Bowen University Teaching Hospital, Ogbomoso, Oyo State, Nigeria, granted approval for this study and all participants involved gave an oral consent.

Plant Materials

Fresh plants of *Basella alba* for the purpose of this study were procured from various humid locations all around Iwo and Lagos, western Nigeria. The plant materials were identified and authenticated in the department of Botany, University of Ibadan with voucher number (UIH-22391). The leaves were separated from their stems, washed in tap water and air dried under shade, without exposure to direct sunlight. The dried leaves were reduced into fine powder by grinding. The aqueous extract was prepared by mixing 100 g of the powdered leaves with 1000 ml of boiling distilled water and the mixture was stirred. Boiling was allowed to continue for 5 minutes. The mixture was kept off the hot plate, for 30 min to allow it to infuse. It was then filtered using cheese cloth and filter paper, to get rid of impurities. The filtrate was then concentrated using a water bath at 60 °C to obtain the solid mass.

Preparation of *Basella alba* Dosages

200mg of the solid mass of *Basella alba* aqueous extract was dissolved in 1000ml of distilled water. Thus, 1ml of this solution is equivalent to a concentration of 0.2mg/ml which is the low dose and 2ml of the solution is equivalent to a concentration of 0.4mg/ml, which is the high dose.

These dose(s) were chosen in accordance to an in-vitro study carried out by Vijender *et al.*, (2011)^[25] on anti-inflammatory activity of leaf extracts of *Basella alba* (methanolic and aqueous extracts), which showed that the aqueous extract at these doses had the most significant red cell membrane stabilizing action.

Experimental Design

15 centrifuge tubes were used for each subject. These were divided into three groups of 5 per group.

The Group 1: served as control, containing 1ml of phosphate buffer, 5ml of varying saline concentrations (0.0%, 0.3%, 0.6%, 0.9% and 1.2%) and a drop of concentrated ammonium hydroxide (to enhance color development and stability). In this group, none of the test tubes contained *Basella alba*.

The Group 2: contained 1ml of phosphate buffer, 5ml of varying saline concentrations (0.0%, 0.3%, 0.6%, 0.9% and 1.2%), concentration of 0.2mg/ml (low dose) of aqueous extract of *Basella alba* in each of the 5 centrifuge tubes at a volume of

1ml and a drop of concentrated ammonium hydroxide (to enhance color development and stability).

The Group 3: contained 1ml of phosphate buffer, 5ml of varying saline concentrations (0.0%, 0.3%, 0.6%, 0.9% and 1.2%), concentration of 0.4mg/ml (high dose) of aqueous extract of *Basella alba* in each of the five centrifuge tubes at volume of 2ml and a drop of concentrated ammonium hydroxide (to enhance color development and stability).

Preparation of Phosphate Buffer

100ml of 0.2M Phosphate buffer was prepared with the use of two salts; disodium hydrogen phosphate (Na_2HPO_4) with molecular weight of 141.96g and sodium dihydrogen phosphate (NaH_2PO_4) with molecular weight of 119.96g.

For disodium hydrogen phosphate, the concentration in mol/dm^3 was multiplied by the molecular weight, to obtain the concentration in g/dm^3 , i.e. $0.2 \times 141.96 = 28.392\text{g}$, approximately 28.4g, thus, 14.2g of Na_2HPO_4 was dissolved in 50ml of distilled water (since 28.4g is to be dissolved in 100ml of distilled water to get a total of 200ml of phosphate buffer).

Likewise, for NaH_2PO_4 , the concentration in mol/dm^3 was multiplied by the molecular weight, to obtain the concentration in g/dm^3 , i.e. $0.2 \times 119.96 = 23.992\text{g}$, approximately 24g, thus 12g of NaH_2PO_4 was dissolved in 50ml of distilled water (since 24g is to be dissolved in 100ml of distilled water to get a total of 200ml of phosphate buffer). Therefore, the addition of 14.2g of Na_2HPO_4 in 50ml of distilled water to 12g of NaH_2PO_4 in 50ml of distilled water, gave a total of 100ml of phosphate buffer.

The pH was adjusted to 7.4 by addition of HCl (Hydrochloric acid) and Sodium hydroxide (NaOH) alternately as necessary to decrease or increase the pH respectively, until it got to 7.4, and this was indicated by the pH meter, which had its electrode placed in the solution.

Preparation of Saline Solutions

Three varying percentages of saline solution were prepared. They include; 0.3%, 0.6% and 1.2%. 0.3% hypotonic solution was prepared by dissolving 0.3g of NaCl (Sodium chloride) in 100ml of distilled water. For 0.6%, 0.6g of NaCl was dissolved in 100ml of distilled water and 1.2g of NaCl was dissolved in 100ml of distilled water to obtain 1.2%. 0.9%.

Collection of Blood Samples and Preparation of Erythrocytes

A volume of 5-6 ml of human venous blood samples was collected by venipuncture, from 12 participants who expressed the HbSS genotype. Blood sample was collected into EDTA anti-coagulant bottles and thoroughly mixed. Erythrocytes were washed 3 times by centrifuging with normal saline at 3000rpm for 10mins, according to Tsakiris *et al.* (2005)^[23], and the test carried out with the washed and intact erythrocytes.

Determination of Erythrocyte Osmotic Fragility

Osmotic fragility of erythrocyte in all samples was determined by a measure of haemoglobin released from red blood cells when placed in an environment containing serial dilutions of Phosphate Buffer Saline (PBS) solution as described by Oyewale (1993)^[21], with minor modifications (Mafuvadze *et al.*, 2008)^[15].

0.1ml of washed red blood cells were suspended in 1.0 ml buffer solution: pH = 7.4 and added to 5ml of the different concentrations of saline i.e. 0.3, 0.5, 0.9 and 1.2 g/100ml of NaCl in different centrifuge tubes. The fifth centrifuge tube contained distilled water. The tubes were then inverted several times to mix its content. The centrifuge tubes were incubated for 30 min at body temperature (37 °C). Subsequently, the contents of test tubes were centrifuged at 1200rpm for 10 min. The supernatant was decanted and haemoglobin content determined spectrophotometrically at $\lambda_{max} = 540$ nm using PBS (0.9 g/100 ml) solution as blank. Haemolysis in each test tube was expressed as a percentage, taken as 100% the maximum value of absorbance of the test tube that contained erythrocytes suspended in distilled water (0.0 g/100 ml).

Evaluation of percentage erythrocyte hemolysis.

The percentage of hemolysis was calculated as follows;
 $\% \text{ of hemolysis} = \frac{\text{Absorbance reading of test supernatant}}{\text{Absorbance reading of 100\% hemolysis}} \times 100$.

The cumulative erythrocyte osmotic fragility curve, of percentage of erythrocyte lysis versus concentrations of saline solution was plotted

Statistical Analysis

All data were presented as mean \pm SEM (Standard Error of Mean). The obtained results were analyzed using the SPSS (20). Student's paired sample t-test was used to compare the results obtained in the control group with that of the low dose and high dose. The results were considered significant at p values of less than 0.05.

Results

Effect of *Basella alba* on In-Vitro Osmotic Fragility in Male Sicklers

The results of the experiment were tabulated as mean \pm standard error of mean. At 0.0%, complete hemolysis (100%) was recorded for control, low dose and high dose of *Basella alba*, and this served as a standard in calculating % hemolysis at other saline percentages (i.e. 0.3%, 0.6%, 0.9% and 1.2%).

Table 1: Percentage of hemolysis in control, low dose and high dose of *Basella alba* at varying saline concentrations in male sicklers. (n=6).

%Saline	%Hemolysis (Control)	%Hemolysis (Low Dose)	%Hemolysis (High Dose)
0.0	100 \pm 0.0	100 \pm 0.0	100 \pm 0.0
0.3	16.91 \pm 3.158	14.22 \pm 3.948	12.26 \pm 2.598
0.6	19.08 \pm 3.937	8.092 \pm 1.838*	6.953 \pm 1.380*
0.9	28.83 \pm 14.52	6.190 \pm 1.165	6.027 \pm 1.446
1.2	19.63 \pm 6.59	4.672 \pm 0.8179*	4.932 \pm 0.6855

*Significant at p<0.05 in comparison with % hemolysis (control).

From the control group, least percentage of hemolysis was seen at 0.3% in this order: 0.0%>0.9%>1.2%>0.6%>0.3%.

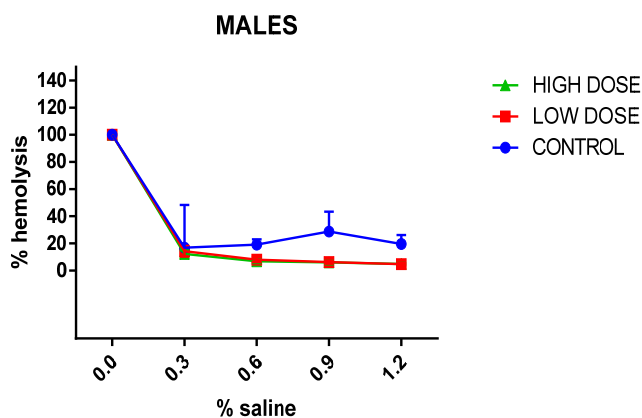


Fig 1: Osmotic fragility curve for control, low dose and high dose of *Basella alba* in male sicklers.

The osmotic fragility curve above represents the % hemolysis versus % saline for control, low dose and high dose of *Basella alba* in male HbSS subjects. Percentage hemolysis of low dose and high dose of *Basella alba*, were low compared with the control group (without *Basella alba*) as seen in FIG 12.

Effect of Low Dose of *Basella Alba* on In-Vitro Osmotic Fragility in Male Sicklers.

At 0.3% saline, a decrease in hemolysis was observed in low dose of *Basella alba* (14.22 \pm 3.948%) compared with the control (16.91 \pm 3.158%), but this was not statistically significant (p>0.05). At 0.6% saline, there was a significant

decrease in hemolysis, in low dose of *Basella alba* (8.092 \pm 1.838%), compared to the control (19.08 \pm 3.937%) (p<0.05). At 0.9% saline, a decrease in hemolysis was observed, in low dose of *Basella alba* (6.190 \pm 1.165%), when compared with the control (28.83 \pm 14.52%) and this was not statistically significant (p>0.05). At 1.2% saline, there was a significant decrease in hemolysis in low dose of *Basella alba* (4.672 \pm 0.8179%) compared with the control (19.63 \pm 6.59%) (p<0.05).

Effect of High Dose of *Basella Alba* on In-Vitro Osmotic Fragility in Male Sicklers

At 0.3% saline, there was a decrease in hemolysis in high dose of *Basella alba* (12.26 \pm 2.598%), compared with the control (16.91 \pm 3.158%) but this was not significant (p<0.05). At 0.6% saline, a significant decrease in hemolysis was observed in high dose of *Basella alba*, (6.953 \pm 1.380%), compared to the control (19.08 \pm 3.937%) (p<0.05). A decrease in hemolysis was observed, in high dose of *Basella alba* (6.027 \pm 1.446%) at 0.9%, compared with the control (28.83 \pm 14.52%) however, this was not significant (p>0.05). Also, the decrease in hemolysis observed at 1.2% in high dose of *Basella alba* (4.932 \pm 0.6855%) compared with control (19.63 \pm 6.59%) was not significant (p>0.05).

Effect of *Basella Alba* on In-Vitro Osmotic Fragility in Female Sicklers

The result of the experiment was tabulated as mean \pm standard error of mean. At 0.0%, complete hemolysis (100%) was recorded, for control, low dose and high dose and this served as a standard in calculating % hemolysis at other saline percentages (i.e. 0.3%, 0.6%, 0.9% and 1.2%).

Table 2: Percentage of hemolysis in control, low dose and high dose of *Basella alba* at varying saline concentrations in female sicklers. (n=6).

% Saline	%Hemolysis (Control)	%Hemolysis (Low Dose)	%Hemolysis (High Dose)
0.0	100±0.0	100±0.0	100±0.0
0.3	18.92±6.092	16.79±2.024	16.06±5.669
0.6	20.07±7.544	10.43±0.9524	12.42±3.405
0.9	9.853±1.832	5.993±1.181	9.728±2.001
1.2	9.620±2.523	8.935±2.128	7.397±1.347

From the control group, least percentage of hemolysis was seen at 1.2% in this order: 0.0%>0.6%>0.3%>0.9%>1.2%.

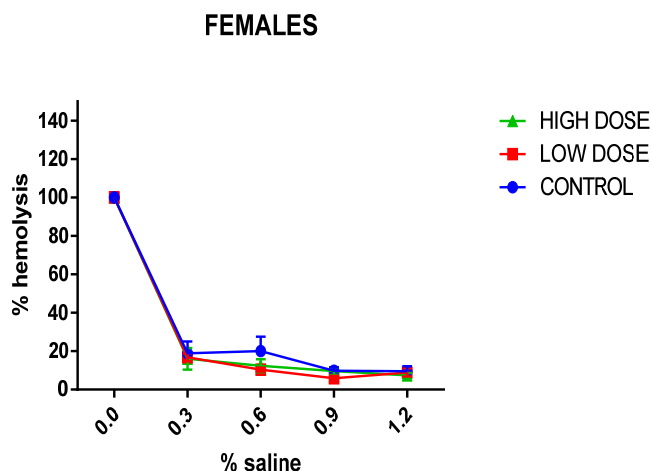


Fig 2: Osmotic fragility curve for control, low dose and high dose of *Basella alba* in female sicklers.

The osmotic fragility curve above represents the % hemolysis versus % saline for control, low dose and high dose in female HbSS subjects. It was observed that low dose and high dose of *Basella alba* reduced hemolysis, compared to the control group (without *Basella alba*).

Effect of Low Dose of *Basella Alba* on In-Vitro Osmotic Fragility In Female Sicklers

At saline concentrations of, 0.3%, 0.6%, 0.9% and 1.2%, low dose of *Basella alba* reduced hemolysis, compared to the control group. However, the difference was not statistically significant (p>0.05).

Effect of High Dose of *Basella Alba* on In-Vitro Osmotic Fragility in Female Sicklers

At saline percentages of, 0.3%, 0.6%, 0.9% and 1.2%, high dose of *Basella alba* reduced hemolysis, compared to the control group. However, the difference was not statistically significant (p>0.05).

Effect of *Basella Alba* on In-Vitro Osmotic Fragility in both Male and Female Sicklers

The result of the experiment was tabulated as mean ± standard error of mean. At 0.0%, complete hemolysis (100%) was recorded for control, low dose and high dose and this served as a standard in calculating % hemolysis at other saline percentages (i.e. 0.3%, 0.6%, 0.9% and 1.2%).

Table 3: Percentage of hemolysis in control, low dose and high dose of *Basella alba* at varying saline concentrations in male and female sicklers. (n=12).

% Saline	%Hemolysis (Control)	%Hemolysis (Low Dose)	%Hemolysis (High Dose)
0.0	100±0.0	100±0.0	100±0.0
0.3	17.91±3.285	15.49±2.146	14.16±3.028
0.6	19.57±4.058	10.63±1.877*	9.683±1.936*
0.9	19.34±7.540	5.841±0.8193	7.423±1.312
1.2	14.62±3.684	6.990±1.243	6.164±0.8108

*Significant at p<0.05 in comparison with % hemolysis (control).

From the control group, least percentage of hemolysis was seen at 1.2% in this order: 0.0%>0.6%>0.9%>0.3%>1.2%.

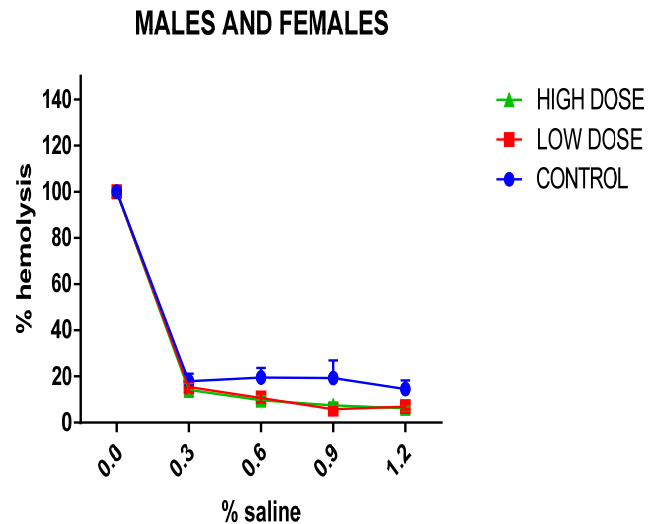


Fig 3: Osmotic fragility curve for control, low dose and high dose of *Basella alba* in both male and female sicklers.

From the osmotic fragility curve above, representing % hemolysis versus % saline for control, low dose and high dose in both male and female HbSS subjects, it was observed that low dose and high dose of *Basella alba*, reduced hemolysis, compared to the control group (without *Basella alba*).

Effect of Low Dose *Basella Alba* on In-Vitro Osmotic Fragility in both Male and Female Sicklers

Decrease in hemolysis was seen in low dose of *Basella alba* at 0.3%, 0.6%, 0.9% and 1.2% compared with the control group. The decrease at 0.3%, 0.9% and 1.2% were not significant (p>0.05), however, at 0.6%, it was significant (p<0.05).

Effect of High Dose *Basella Alba* on In-Vitro Osmotic Fragility in both Male and Female Sicklers

Decrease in hemolysis was seen observed in high dose of *Basella alba* at 0.3%, 0.6%, 0.9% and 1.2% compared with the control group. The decrease at 0.3%, 0.9% and 1.2% were not significant (p>0.05), however, at 0.6%, it was statistically significant (p<0.05).

Discussion

The in-vitro effect of aqueous extract of *Basella alba* leaves on red blood cell fragility in sicklers was examined in this study. The actual anemia of the illness is caused by hemolysis; the destruction of red blood cells because of their shape, thus the

sickled cells only last 10-20 days, as opposed to healthy red blood cells that typically function for 90-120 days (Joseph, 2014) [9], hence, hemolysis was observed even in isotonic solution (0.9%), where normal red cell membrane maintain their integrity and also there was hemolysis in hypertonic solution (1.2%) in all subjects, owing to the hemolytic potentials of the sickling red blood cells.

In male sicklers, higher percentages of hemolysis were observed in the group not treated with *Basella alba* when compared to that seen in female sicklers. This is because Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency (a condition that predisposes to hemolysis) mostly affects males of African or Mediterranean descent (Chan, 1996) [4].

Low dose of *Basella alba* however reduced hemolysis significantly at 0.6% and 1.2% saline and high dose of *Basella alba* reduced hemolysis significantly at 0.6% saline. This shows that *Basella alba* reduces hemolysis significantly in hypotonic solution (0.6%), as well as in hypertonic solution (1.2%). Although, under normal circumstances, crenation of red blood cells is to be observed in hypertonic solution(1.2%), in which the red cell loose water and shrinks, however, due to the structure of the sickle erythrocytes, hemolysis was observed, and low dose of *Basella alba* reduced this hemolysis significantly.

Generally, males have a higher percentage of androgens, compared to females, particularly testosterone, which increases the output and effectiveness of erythropoietin, that in turn stimulates erythropoiesis (Frascino, 2002) [7] thus increasing red blood cell count in males (4.7-6.1 million/mcL) compared to 4.2-5.4 million/mcL in females (Amber, 2015) [2]. Considering the fact that *Basella alba* extract has demonstrated androgenic potential in adult rats and bull Leydig cells (Moundipa *et al.*, 2005) [17], this suggests that there might be a possible synergistic activity between *Basella alba* and androgens, by which *Basella alba* reduces hemolysis of red blood cells, combined with its anti-anemic effect, which was proven in an earlier discovery by Bamidele *et al.*, (2010) [3] and aids to maintain the high red blood cell count seen in males. The mechanism by which this occurs is open to further research.

In female sicklers lower percentages of hemolysis was seen, compared to that observed in the males this is probably due to the decreased red blood cell count seen in females. Although, both low dose and high dose of *Basella alba* decreased hemolysis, this was not statistically significant.

In both male and female sicklers, a significant decrease in hemolysis was observed when low dose and high dose of *Basella alba* was administered to red blood cells placed in hypotonic solution (0.6%), where the highest percentage of hemolysis was observed. The effect of *Basella alba* on osmotic fragility is seen to be dose-related because, the low dose of *Basella alba* reduced hemolysis in both male and female sicklers (especially males), and high dose of *Basella alba* reduced hemolysis more, although it was only statistically significant at 0.6% saline.

Conclusion

Aqueous extract of *Basella alba* L. leaves extracts exhibited reduction in hemolysis of sickle red blood cells by inhibiting hypotonicity-induced lysis of erythrocyte membrane, significantly at 0.6% saline in both male and female sicklers, as well as reducing hemolysis significantly in hypertonic solution in males. Thus *Basella alba* can be said to help reduce the

occurrence of hemolytic crisis in sicklers, and consequently help in extending the lifespan of their red blood cells, thereby compensating for the inability of their bone marrow to keep up with the production of new red blood cells, because their red blood cells get destroyed faster than normal. *Basella alba* thus reduces the occurrence of hemolytic anemia in subjects with sickle cell anemia, via its possession of antioxidants that further help to stabilize the red cell membrane, hence, reducing lysis. It is therefore, recommended for consumption as part of daily diet of both male and female sicklers.

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