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Short Communication

Effect of *Hibiscus Sabdariffa* Calyx Extract on Derived Haematologic Indices in Sickle Cell Anaemia *In-vitro*

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ABSTRACT

We hypothesised that the calyx extract of Hibiscus sabdariffa (HS) may have antisickling potential on account of its calcium antagonistic and antioxidants effects. Five ml of blood was collected from sickle cell anaemia (SCA) patients (n=11). 50µL of blood was incubated with 50µL each of 0.l, 1 and 10mg/ml of HS before adding 50µL of 2% sodium metabisulphite (Na2S2O5) (protective assay). Also, 50µL Na2S2O5 was added to 50µL of blood and then incubated with 50µL each of 0.l, 1 and 10mg/ml of HS (reversal assay). In parallel, 50µL of blood each was incubated with 50µL of 5 mg/ml hydroxyurea and 50µL of 0.9% NaCl (control) respectively. The mean cell haemoglobin concentration (MCHC), mean cell volume (MCV) and mean platelet volume (MPV) were determined. In both the protective and reversal assays, a significant ($p<0.01$ and $p<0.05$ respectively) decrease in MCHC was observed in the presence of HS. However, there was a significant ($P<0.05$) increase in MCV in the presence of HS in the reversal assay only. In the protective and reversal assays the MPV increased significantly ($P<0.05$ and $P<0.01$ respectively) in the presence of HS. These parameters showed no significant change in the presence of hydroxyurea. These results suggest that in the reversal assay HS may have antisickling properties by lowering the MCHC and increasing MCV and thereby reducing haemoglobin S concentration and polymerization. This further suggests that HS may have a Gardos channel inhibitory effect. However, the increase in MPV suggests that HS may be toxic at these concentrations.

Keywords: *Antisickling effect, Hibiscus sabdariffa calyx extract, MCHC, MCV, MPV, Gardos channel blocker.*

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INTRODUCTION

Sickle cell disease (SCD) is a group of haematologic disorders, including homozygous sickle cell anaemia (SS), sickle alpha-thalassemia disease, sickle beta-thalassemia disease, sickle haemoglobin C disease (SC), sickle hereditary persistence of foetal haemoglobin (S/HPFH) and other less common variants (Bender, 2003). Nigeria probably has the highest number of SCD sufferers in the world because of her population and location (WHO, 2005). Following the success of hydroxyurea in reducing painful crises in a double-blind placebo-controlled randomized clinical trial, (Charache *et al*., 1995; Charache, 1997) it became the first drug to be approved for the treatment of sickle cell anaemia. However, hydroxyurea causes bone marrow depression. Hence its use must be accompanied by frequent blood counts, which may not be affordable or easily achievable in rural Africa where most patients reside. In addition, as a cytostatic agent, there are fears about potential carcinogenic or leukemogenic effects following prolonged usage (Charache *et al*, 1995; Jinna *et al*, 2020). Despite these, hydroxyurea is widely used in Europe and America, where careful monitoring of its dose is possible thereby keeping the dangerous side-effects at bay.

Clearly, there is the need to search for less toxic alternatives. A potential source of these may be the rich repertoire of medicinal plants in Africa. One of such plants may be the calyces of *Hibiscus sabdariffa* (HS; family: Malvaceae). HS has been shown to cause vasodilation (Owolabi *et al*, 1995; Adegunloye *et al*, 1996) by antagonizing Ca^{2+} entry into vascular smooth muscle cells (Owolabi *et al*, 1995; Alsayed *et al*, 2020). Since sickling is Ca^{2+}-dependent, it is conceivable that HS may prevent sickling by inhibiting Ca^{2+} influx into sickle cells. In addition, SCD is associated with oxidative stress (Akohoue *et al*, 2007; Antwi-Boasiako *et al*, 2019) and HS has antioxidant properties (Usoh *et al*, 2005; Hirunpanich *et al*, 2006). It is conceivable that HS may also quench the oxidative stress on account of its antioxidant action. Consequently, this study tested the hypothesis that HS

may possess antisickling properties by inhibiting calcium influx into sickle cells and by quenching the oxidative stress in SCD. To test this hypothesis, we examined the effect of HS on erythrocyte indices of sickle cell anaemia patients *in vitro*.

MATERIALS AND METHODS

Study Design: The study was a laboratory-based experiment involving quantitative analysis of blood samples from stable sickle cell anaemia patients that are not on hydroxyurea medication.

Plant extraction procedure: Calyces of *H. sabdariffa* (200g) were soaked in two litres of deionised water and then placed into a water bath set at 40^{o}C for about five hours. After five hours, the calyces were sieved off remaining the extract. The extract was then evaporated in an electric oven set at 60^{o}C till a completely dried extract was obtained. The extract was then divided into small aliquots and kept in the refrigerator at a temperature of 4^{o}C till it was used.

Laboratory Preparation and Experimentation: Following ethical approval with reference number UDUTH/HREC/2014/NO191 from the ethical committee of the Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto, Nigeria and informed consent, five ml of venous blood was collected into EDTA bottles from sickle cell anaemia patients (n=11) in the stable state. Sickle cell disease patients other than homozygous SS patients were excluded from this study.

Baseline erythrocytic indices such as mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), and mean platelet volume (MPV) were measured using a full haematology autoanalyzer (Mythic 22CT, Orphée, Switzerland). The blood sample was then divided into four groups and experiments were carried out as described below.
To the first group which was the protective assay, 50µL of blood each was incubated with equal volume of *H. sabdariffa* extract (0.1 mg/ml, 1 mg/ml and 10 mg/ml in normal saline) for 30 minutes at 37^{o}C. Then these were challenged with 50µL of 2% sodium metabisulphite for 20 minutes (Cheesbrough, 2001). Thereafter, haematological parameters such as MCHC, MCV and MPV were measured using a full haematology autoanalyzer.

In the second group, which was the reversal assay, 50µL of blood each was sickled with 50µL of 2% sodium metabisulphite for 20 minutes. To these aliquots were added 50µL of HS extract (0.1 mg/ml, 1 mg/ml and 10 mg/ml in normal saline). The aliquots were incubated for 30 minutes at 37°C in a water bath. The MCHC, MCV and MPV of the samples were then measured.

In the third group, 50µL of blood was incubated with 50µL of hydroxyurea (5mg/ml), while in the fourth group 50µL of blood was incubated with 50µL of normal saline (NS).

Statistical Analysis

The results are presented as mean±SEM. They were analysed using one-way ANOVA with a post-hoc Dunnett's multiple comparison test by means of GraphPad Instat statistical software. $P < 0.05$ was regarded as statistically significant.

RESULTS

The results from this study are presented in Table 1.

Effect of *Hibiscus sabdariffa* on mean cell haemoglobin concentration (MCHC): Table 1 shows results of the protective assay for MCHC. No significant difference was observed in MCHC at 0.1 mg/ml and 1 mg/ml concentration of HS when compared with the control group (blood + normal saline), hydroxyurea and blood only. However, the MCHC fell significantly ($P<0.01$) at the HS concentration of 10mg/ml when compared to control, hydroxyurea and blood only.

In the reversal assay the MCHC fell significantly at the HS concentrations of 0.1mg/ml and 10mg/ml ($P<0.05$ each) compared to the control group (blood + normal saline), hydroxyurea group and blood only. However, it did not differ significantly in the 1mg/ml HS group compared to these groups.

Effect of *Hibiscus sabdariffa* on mean cell volume (MCV): Table 1 also shows the results of the protective assay for MCV. In this assay, the MCVs of 0.1 mg/ml, 1 mg/ml and 10 mg/ml HS showed no significant difference from the control MCV (blood + NS) and those of hydroxyurea and blood only. Though the MCVs of 0.1mg/ml and 1mg/ml HS in the reversal assay did not differ significantly from the MCV of the control group, hydroxyurea and blood only, it increased significantly ($P<0.05$) in the 10mg/ml HS group compared to these groups.

Table 1:
The effect of graded concentrations of the calyx extract of *Hibiscus sabdariffa* (HS) on the mean cell haemoglobin concentration (MCHC), mean cell volume (MCV) of the red blood cells and mean platelet volume (MPV) of whole blood incubated with it compared to whole blood alone and whole blood incubated with normal saline (NS) and hydroxyurea (HU) (Protective Assay). Data are expressed as Mean ± SEM. (n= 11)

Analyte	MCHC (g/dL		MCV (fL)		MPV (fL)	
	Protective assay	Reversal Assay	Protective assay	Reversal Assay	Protective assay	Reversal Assay
Blood only	36.90 ± 0.30	36.90 ± 0.30	81.63 ± 2.11	81.63 ± 2.11	8.17 ± 0.23	8.17 ± 0.23
Blood + NS (control)	37.76 ± 0.35	37.76 ± 0.35	80.72 ± 2.16	80.72 ± 2.16	8.41 ± 0.2	8.41 ± 0.2
Blood +5.0mg/ml HU	36.93 ± 0.33	36.93 ± 0.33	81.09 ± 2.07	81.09 ± 2.07	8.67 ± 0.39	8.67 ± 0.39
Blood + 0.1mg/ml HS	36.64 ± 0.47	34.98 ± 0.61*	82.81 ± 2.08	84.48 ±2 .26	9.75 ± 0.35*	11.51±0.37*
Blood + 1.0mg/ml HS	36.94 ± 0.47	35.22 ± 0.65	83.06 ± 1.95	83.57 ± 2.02	9.71 ± 0.35*	11.44±0.32*
Blood +10.0mg/ml HS	31.57± 1.65*	33.08 ± 2.08*	96.44 ± 5.72	96.51 ± 3.38*	9.63 ± 0.33	10.82±0.5*

**= Significant at P < 0.05, when compared with blood + NS (control), hydroxyurea and blood only*

Effect of *Hibiscus sabdariffa* on mean platelet volume (MPV): HS had a significant increase in MPV in the 0.1mg/ml and 1mg/ml of HS groups ($P<0.05$) respectively when compared to the MPV of blood + normal saline (control group), hydroxyurea and blood only. However, there was no significant difference in the MPV of 10mg/ml HS group compared to these groups. The MPV of blood treated with 0.1 mg/ml, 1 mg/ml and 10 mg/ml HS increased significantly ($P<0.01$ respectively) compared to the MPV of blood + normal saline (control), hydroxyurea and blood only in the reversal assay.

DISCUSSION

We demonstrated that HS has antisickling properties by its ability to decrease MCHC, with a corresponding increase in MCV in the reversal assay. This suggests that HS might have the ability to increase the hydration of sickle cells and consequently lower deoxy Hb S concentration as evidenced by the increase in MCV and decrease in MCHC respectively. This implies that it has the ability to lower the concentration deoxy Hb S and thereby inhibit the polymerization of HB S and ultimately sickling. HS also increased MPV in both reversal and protective assays suggesting that it may be toxic at the concentrations used.

The approaches to prevent intravascular sickling may be broadly divided into those preventing the gelation of HbS (i.e inhibit HbS polymerisation) that occur when there is deoxygenation; those that modify the red cell membrane making it less susceptible to sickling; and those improving peripheral perfusion (Serjeant and Serjeant, 2001). Approaches to prevent or inhibit HbS polymerisation include: blocking intermolecular sickle cell fibre contacts, stimulation of HbF synthesis, increased oxygen affinity, reduction of 2,3-diphosphoglycerate concentration or reduction of Hb concentration in the cells (Eaton and Bunn, 2017). Studies have shown that one of the mechanisms of action of hydroxyurea; a medication used in the management of sickle cell disease is by increasing the level of circulating foetal haemoglobin (Charache *et al*, 1995; Charache 1997; Steinberg *et al*, 1997; Steinberg 1999). Hydroxyurea-induced foetal haemoglobin level tends to obstruct HbS polymerisation by preventing contacts with other HbS molecules, in addition to forming HbS hybrids that have higher solubility than Hb S (Halsey and Roberts, 2003). Furthermore, foetal haemoglobin has a higher oxygen affinity (McCarthy 1943), which probably causes a decrease in the concentration of deoxy-HbS and mean cell haemoglobin concentration thereby preventing haemoglobin S polymerization and sickling. Nevertheless, Hydroxyurea showed no antisickling properties when compared to the control group in this study. One probable reason could be due to the *in vivo* mechanism of action of hydroxyurea on increasing circulating level of foetal haemoglobin which might not be feasible in *in-vitro* studies. Further studies are needed to ascertain this.

The results of this study showed that HS caused a decrease in MCHC in both assays with a corresponding increase in MCV in the reversal assay only. It is not clear how HS was able to reduce MCHC in the protective assay without a corresponding increase in MCV. One explanation for this could be that HS probably increased the affinity of haemoglobin S for oxygen thereby indirectly lowering the concentration of deoxy haemoglobin S which results in a reduced MCHC. Actual experiments are needed to be done to ascertain this notion.

The lowering of MCHC and the corresponding increase in the MCV of sickle cells in the reversal assay suggests that these cells have become more hydrated in the presence of HS. This further suggests that HS may have acted as a Gardos channel blocker. The Gardos channel is a channel on the red cell membrane that allows Ca^{2+} -activated K^+ efflux (Gardos 1958; Stuart *et al*, 1994) thereby, leading to the dehydration of the sickle cell, a fall in MCV and a corresponding increase in MCHC. These lead to an increase in deoxy haemoglobin S concentration and a resultant haemoglobin S polymerization and sickling (Stuart *et al*, 1994). HS may be able to block the Gardos channel on account of its inhibition of Ca^{2+} influx (Owolabi *et al*., 1995; Alsayed *et al*, 2020) thereby preventing K^+ efflux, making the sickle cells well hydrated and increasing the red cell volume (MCV). This results in the lowering of the concentration of deoxy haemoglobin S thereby preventing its polymerization and sickling (Stuart *et al*, 1994). In addition to this, HS has been shown to have antioxidant properties (Usoh *et al*, 2005; Hirunpanich *et al*, 2006) and could have exerted its antioxidant effect on the red blood cells, since sickle cell anaemia is associated with oxidative stress (Hebbel *et al*, 1982; Klings *et al*, 2001; Klings and Farber, 2001; Akohoue *et al*, 2007). Apparently, findings from this study have indicated for the first time the antisickling potentials of HS on MCHC and MCV. One may possibly say that aqueous calyx extract of HS could be a valuable source of antisickling agents. The results from the present study also further confirm the beneficial roles of phytochemicals in antisickling activity. Thus, medicinal plants with rich phytochemicals could be of great value in the management of SCA, as has been shown by other investigators in plants like *Carica papaya* (Oduola *et al*, 2006), *Fagara zanthoxyloides* (Imaga, 2010), *Garcinia kola Heckel* (Adejumo *et al*, 2011), *Hymenocardia acida* (Ibrahim *et al*, 2007) and *Moringa oleifera* (Adejumo *et al*, 2012).

HS also caused an increase in mean platelet volume (MPV). High levels of MPV (Khandekar *et al*, 2018) and platelet distribution width (Amin *et al*, 2004) have been reported in sickle cell anaemia. Increased MPV values may be used as a marker of vaso-occlusive crises (Khandekar *et al*, 2018) and a predictor of cerebrovascular events (Celik *et al*, 2015) in SCD patients. One reason for this could be as a result of elevated platelet activation which results in vaso-occlusion during sickle cell crisis (Khandekar *et al*, 2018). Furthermore, available evidence suggests that MPV may be useful as a prognostic indicator in people suffering from cardiovascular disease (Chu *et al*, 2010). Indeed, increase in MPV has been shown to be a predictive indicator of cerebrovascular risk (Vizioli *et al*, 2009) and cardiovascular disease (Chu *et al*, 2010). Although the reasons for the elevation in MPV of HS treated group, as seen in this study, remain unclear, the weight of evidence, as presented above, suggests that increased MPV by HS is a toxic side effect. However further studies, both in vitro and in vivo, are needed to confirm the observations of the present study.

In summary, this study revealed that the calyx extract of *Hibiscus sabdariffa* had a significant decrease in mean cell

haemoglobin concentration (MCHC) and an increase in mean cell volume (MCV) in the reversal assay suggesting that it may be a Gardos channel blocker. It is concluded that HS could be a source of antisickling agents for the management of SCA although the concentrations used in this study may be toxic

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