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The Aqueous Extract of *Hibiscus sabdariffa* L. Calyces Effectively Corrects Induced Anemia

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ABSTRACT

Background: Medicinal plants are used by different people around the world to solve health problems. The challenge today is to scientifically prove their efficacy and safety. This work was aimed to experimentally evaluate the therapeutic efficacy of *Hibiscus sabdariffa* L. (Malvaceae) calyces extract used in Benin to treat anemia. **Methods: P**hytochemical screening of *Hibiscus sabdariffa* calyces aqueous extract was prepared. Wistar rats were made anemic by injecting phenylhydrazine for two days. The rats were then treated for two weeks either with distilled water or vitafer (anti-anemic drug) or extract at 200 or 300 mg / Kg body weight. Blood samples were collected on different days for blood count and to analyze the red blood cells osmotic resistance. **Results:** The phytochemical screening revealed presence of catechin tannins, galiques tannins, flavonoids, anthocyanins, leucoanthocyanin, quinone derivatives, terpenoids, steroids, reducing compounds, mucilage, anthracene derivatives and free o-glycoside. The extract corrected anemia in a week by stimulating the synthesis of hemoglobin and the production of red blood cells with a dose-dependent effect. It induced a release of immature red blood cells (microcytosis and hypochromia) only the first week of correction. The extract did not change significantly the number of blood platelets during the experiment suggesting specificity on erythroid lineage. **Conclusion:** The aqueous extract of *Hibiscus sabdariffa* calyces counteract induced anemia with specificity and in a dose-dependent manner.

INTRODUCTION

Anemia is a common blood disorder that affects people of all ages, although the people at greater risk are the elderly, young women of child-bearing age and the infants. According to the WHO, the global prevalence of anemia in the word is 24.8% and estimate to 1620 million people (de Benoist et al. 2008). In anemia there is decreased level of circulating hemoglobin, less than 13 g/dl in male and 12 g/dl in females (Okochi et al. 2003). In the tropics, due to endemicity of malaria, between 10 to 20% of the population presents less than 10 g/dl of Hemoglobin (Diallo et al. 2008). Iron deficiency anemia is common in poor countries because most of the people are suffering from malnutrition. It occurs when the body does not have enough iron, leading to the decreased production of red blood cells because iron is key factor for hemoglobin synthesis (Al-Zabedi et al. 2014). In women, at their reproductive age anemia occurs due to menorrhagia and in pregnancy, it is due to excess need of iron (Ramesh et al, 2010; Rajarathinam et al, 2013). Iron supplementation is mainly used for the anemia management (Sembulingam et al, 2007). In our regions, traditional medicines have several plants to manage anemia.

Hibiscus sabdariffa L. (Malvaceae) is a species of hibiscus which grows in world tropics. The plant can be found in almost all warm countries of Africa, America and Asia (Mat et al. 1985; Rao, 1996; Abu-Tarboush et al. 1997; Cheworin et al. 1999). In these regions, the seeds are used for nutrition, whereas other parts are used for its medicinal properties (Da-Costa-Rocha et al. 2014). It treats hypertension and hyperlipidemia (Hopkins et al, 2013) and the seeds are also used as antianemic in Soudan (Ahmed et al. 2013). In Benin, calyx based drinks of the plant are administered in traditional medicine to treat anemia. The aim of present study was to test the aqueous extract of the plant calyces on anemic *Wistar* rats.

MATERIALS AND METHODS

Animals

Animal material consisted of *Wistar* albino rats of average body weight 185 g, having free access to water and food and acclimated to farming conditions from the pet of the Research Laboratory in Applied Biology (LARBA) located at the Polytechnic School of the University of Abomey (EPAC) in the Benin Republic. Breeding was done in a well-ventilated room, with a day-night rhythm of 12 h. The animals were kept in wire mesh cages with metal feeders and drinking troughs. Their daily diet was made from a mixture of food in the form of

croquettes and marketed by Vet Services (Benin). The enclosure was regularly cleaned to ensure optimal development of the animals avoid infection.

Identification and Preparation of Plant Materials

Hibiscus sabdariffa L. (Malvaceae) calyces were collected from N'Dali in Benin during April 2013. The collected samples were identified and authenticated at the National Herbarium of Benin (HNB) at the University of Abomey Calavi. The samples were dried at moderate temperatures (20-25^0 C), protected from moisture for four weeks. They were then crushed into powder and stored in suitable containers at room temperature. 50 g of the powder was boiled in 500 ml of distilled water contained in a 1000 ml flask for 30 minutes. After cooling the filtrate collected is evaporated in a rotary evaporator between 50° C and 60° C. The extract was dried in an oven at 50° C. The dry residue obtained was powdered and kept in the refrigerator in a black bottle.

Phytochemical Screening of *Hibiscus sabdariffa* calyces extract

Screening was a qualitative chemical analysis based on differential staining reactions and/or precipitation of the major chemical compounds groups contained in plants. The experimental methodology adopted in this study was that of Houghton et al. (1998). The targeted compound were alkaloids, phenolic compounds, tannins, catechin tannins, gallic acid, tannins, flavonoids, anthocyanins, leucoanthocyanin, quinine derivatives, saponosides, triterpenoids, steroids, cardenolides, mucilage, coumarins, reducing compounds and anthracene derivatives.

***In vivo* Experimentation**

The evaluation of the anti-anemic activity consisted of assessing the impact of *Hibiscus sabdariffa* calyces aqueous extract on hematological parameters and red blood cells osmotic resistance of anemic female and male *Wistar* rats.

Induction of Anemia

Anemia was induced by phenylhydrazine hydrochloride. Phenylhydrazine was previously dissolved in a DMSO solution diluted to one-tenth in distilled water. It was administered to rats intraperitoneally (IP) at a dose of 40 mg/kg of body weight/day (Naughton BA et al., 1995) for two days (D0 and D1).

Experimental Protocol

Five groups each having five rats were formed. Group 1 was not anemic and served as control. The rats of other groups were anemic. Groups 3, 4 and 5 were treated with either the vitafer or extract at 200 or 300 mg/kg of body weight/day from D2 to D15. The extract and vitafer were administered by gavage using a gastric tube. Vitafer is reference drug commonly use to treat anemia. The details of the protocol is presented as follows:

Group 1: non-anemic control, consisting of rats given the DMSO diluted one tenth with distilled water on D0 and D1 and then distilled water only on D2 to D15.

Group 2: anemic control consisting of rats given the phenylhydrazine at 40 mg/kg/day for two days (D0 and D1) and distilled water from D2 to D15.

Group 3: Control reference, made of rats given the phenylhydrazine at 40 mg/kg/day for two days (D0 and D1) and 1 ml/kg/day of vitafer, from Day 2 to D15.

Group 4: Rats were given the phenylhydrazine at 40 mg/kg/day for two days (D0 and D1) and 200 mg / kg /day of the *Hibiscus sabdariffa* calyces aqueous extract from D2 to D15.

Group 5: Made of rats given the phenylhydrazine at 40 mg/kg/day for two days (D0 and D1) and 300 mg/kg/day of the *Hibiscus sabdariffa* calyces aqueous extract from D2 to D15.

Blood tests

Approximately 2 ml of blood samples were collected in EDTA tube on days: D0, D2, D7, D10 and D15 by orbital puncture after anesthesia rats with chloroform. They were used for the determination of the blood count and osmotic resistance of red blood cells.

- **Blood Count**

Hematological parameters such as hemoglobin, the number of red blood cells, mean corpuscular volume and mean corpuscular hemoglobin concentration number of platelets were determined with PLC SYSTEM KX 21.

- **Osmotic Resistance of Erythrocytes**

The test was based on the ability of red cells to resist to hemolysis in a hypotonic solution. Blood was diluted 1/200 in two salt solutions of different concentrations. One was isotonic

(0.9% NaCl) and the other hypotonic (0.45% NaCl). Red cells were counted with a Malassez cell. The ratio of the number of red blood cells counted in the hypotonic solution over that of the isotonic solution was the percentage of red blood cells resistant to hemolysis. This test was used to assess the production of young red blood cells.

Statistical Analysis

Graphs were plotted using Graphpad software. In each group, the different means were compared to that of D0 using ANOVA one way, Dunnett's Multiple Comparison Test. The significance level was set at 5%.

RESULTS

Main chemical groups identified in *Hibiscus sabdariffa* calyces aqueous extract

The phytochemical screening of the aqueous extract of *Hibiscus sabdariffa* calyces revealed catechin tannins, galiques tannins, flavonoids, anthocyanins, leucoanthocyanin, quinone derivatives, terpenoids, steroids, reducing compounds, mucilage, anthracene derivatives and free o-glycoside (Table 1).

Table 1: Phytochemical screening of *Hibiscus sabdariffa* calyces extract

Compounds	Détection
Alkaloids	-
Catechic tannins	+
Gallic Tannins	+
Flavonoids	+
Anthocyanins	+
Leucoanthocyanate	+
Quinone derivatives	+
Terpenoids	+
Steroids	+
Cardenolides	-
Saponosides	-
Cyanogenic derivatives	–
Reducing compounds	+
Mucilages	+
Coumarins	-
Free anthracene derivatives	+
C-heteroside	-
O-heteroside	+

Legend: Positive = +; Negative = -

The extract treats anemia in a dose-dependent manner

Anemia is indicated by the hemoglobin level. The mean hemoglobin ranged from 12.2 to 15.1 g/dl in groups of rats at day 0 (D0). It decreased significantly in D2, following hemolysis induced by phenylhydrazine and its value was between 7.1 and 7.5 g/dl. This decrease was corrected at D7 by vitafer or 300 mg extract/kg and at D10 by the extract at 200 mg/kg, indicating a dose-dependent effect of the extract. At Day 15, the mean hemoglobin significantly exceeded the value of D0 with the extract at 300 mg/Kg. The mean hemoglobin level did not change significantly in the group of non-anemic rats (control group) throughout the experimental period (Figure 1).

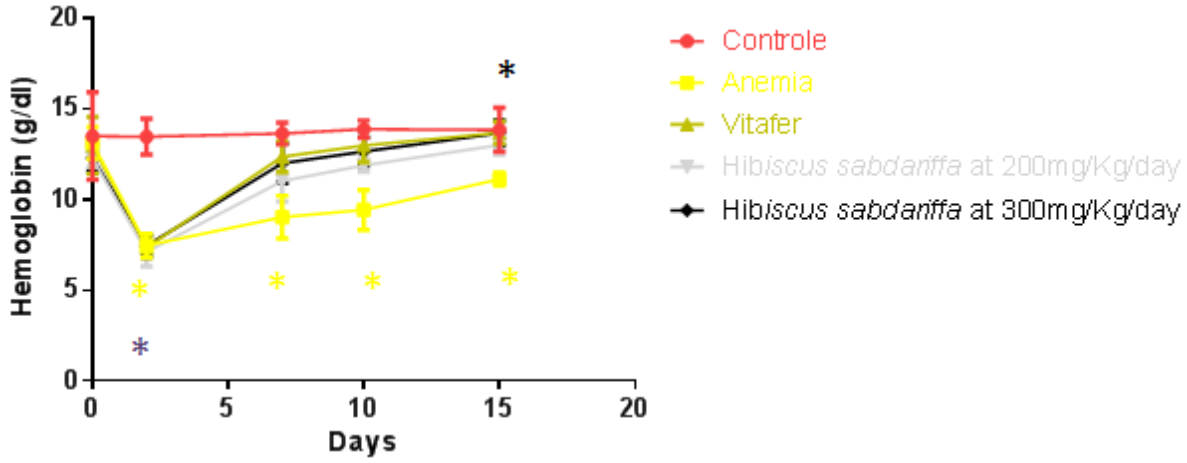

Figure 1: Hemoglobin levels in different groups

The extract correct anemia by stimulating erythropoiesis

Hemoglobin is synthesized by the red blood cell. The mean number of red blood cells varies from 4.0 to 4.4 t/l to D0. It collapsed following hemolysis induced by phenylhydrazine and had a value between 2.9 and 3.0 T/ l in the different groups of rats at Day 2. This decrease was corrected at D7 in groups treated with vitafer or extracts at 200 or 300 mg/kg and only at D10 in untreated anemic the group of rats. On D15, the number of red blood cells significantly exceeded its D0 value in the groups treated with vitafer or 300 mg extract/kg, indicating dose-dependent effect of the extract. The mean number of red blood cells did not change significantly in the group of non-anemic rats (control group) throughout the experimental period (Figure 2).

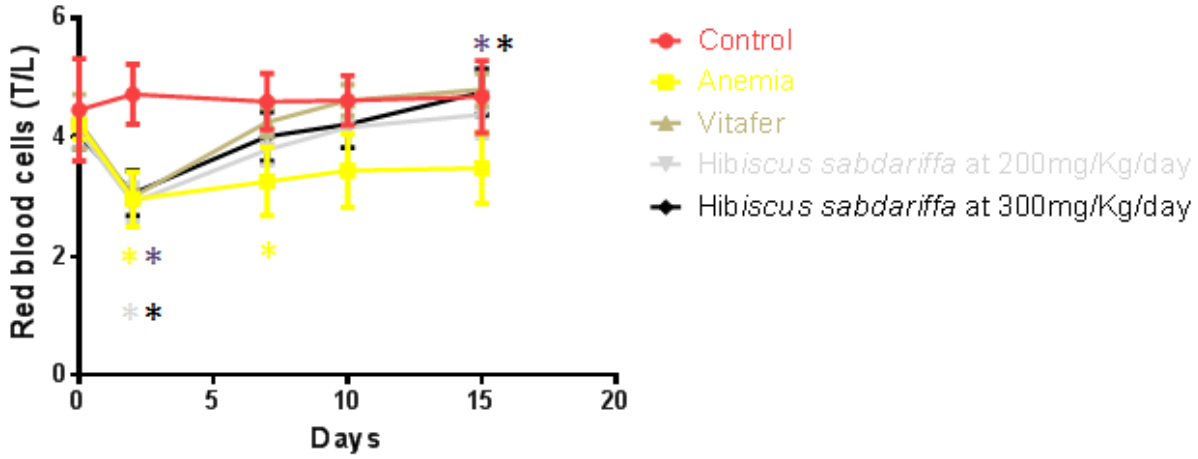

Figure 2: Number of red blood cells in different groups

The extract the correct cell volume

Mean corpuscular volume ranges from 82-85 fl at D0 in different groups of rats. It declined at Day 2 after the administration of phenylhydrazine and varies from 70 to 74 fl. It quickly found its normal value at D7 in the groups treated with the extract or vitafer and significantly exceeded the value of D0 in the group treated with 200 mg extract / kg, indicating a release of macrocytes in blood. It then fell from D10 in the treated groups, indicating a release of more mature red cells in the bloodstream. In the untreated anemic group, the cell volume will return to normal at day 15 (Figure 3).

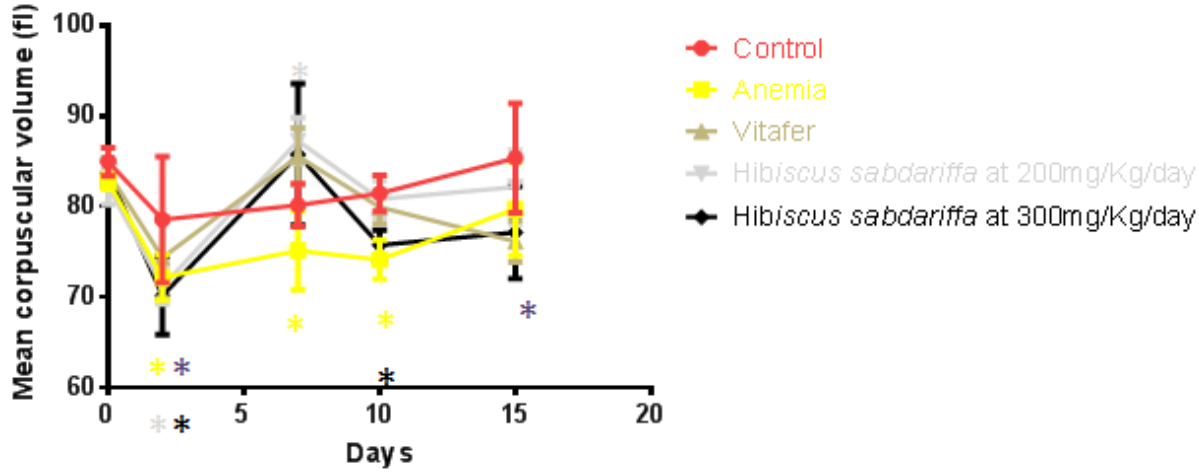

Figure 3: Mean globular volume in the different groups

The mean corpuscular hemoglobin concentration and the cell volume move inversely

Mean corpuscular hemoglobin concentration ranged from 35.4 to 36.4 pg in the various groups of rats at D0. It gradually diminished in the anemic group and this decrease was significant at D7 in the untreated group and in those treated with vitafer or extract at 200 mg/Kg, indicating hypochromia. This decrease was corrected as soon D10 in the treated groups and D15 only in the untreated anemia (Figure 4).

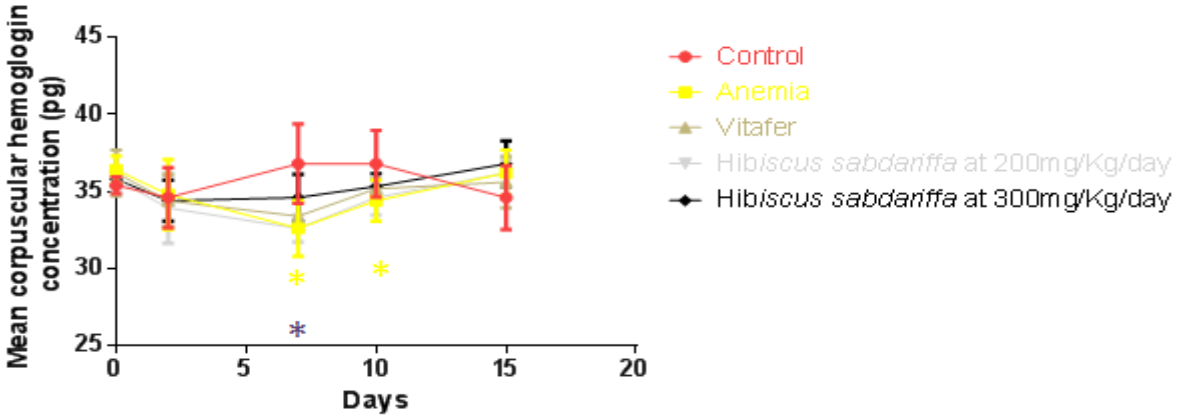

Figure 4: Mean corpuscular hemoglobin concentration in the different groups

Anemia is counteracted by an early release of young red cells in the blood

The osmotic resistance of erythrocytes reflects the proportion of young red cells in the blood. It varied from 21 to 30% in all groups at D0. It increased quickly in all anemic groups and peaked (60-71%) at D7. In the group of non-anemic rats, it first fell slightly at Day 2 (10%) and then gradually increased to J15 (figure5).

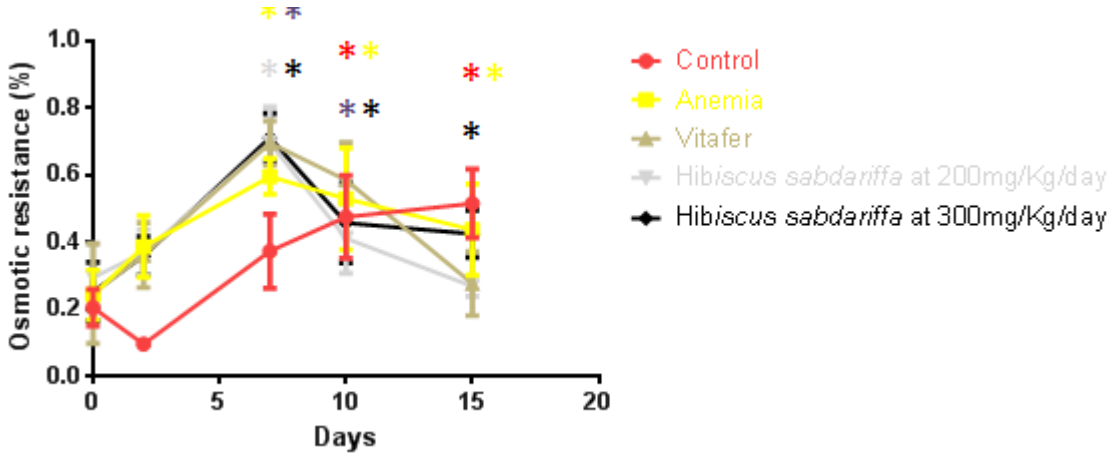

Figure 5: Progression of the red blood cells osmotic resistance in the different groups

Compensation anemia does not affect the rate of blood platelets

The mean rate of blood platelets varies from 484-591 G/l in the various groups of rats at D0. This rate displayed significant changes only in the untreated anemic group of rats during the experimental period, indicating that the extract did not stimulate platelets lineage.

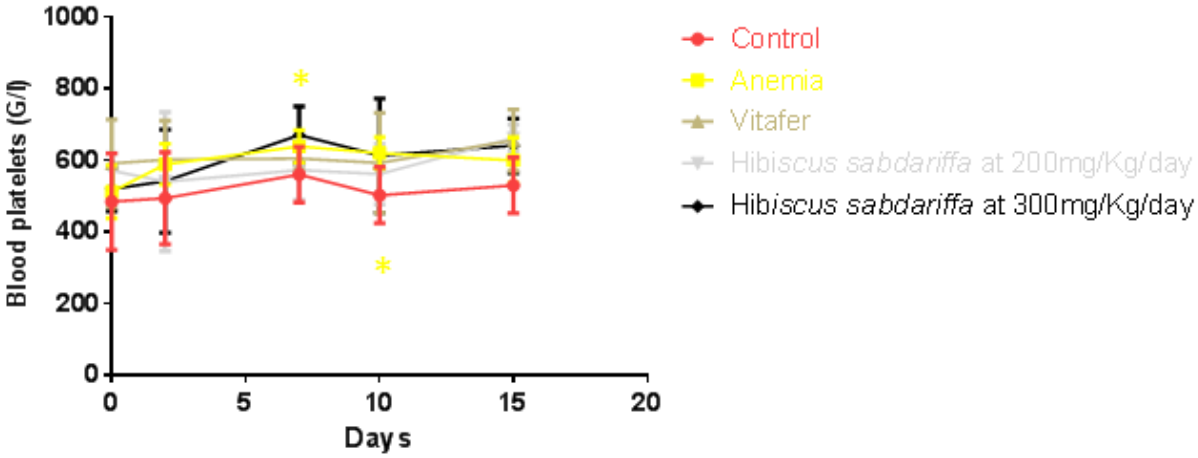

Figure 6: Changes in the number of blood platelets in the different groups

DISCUSSION

Hibiscus sabdariffa L. (Malvaceae) is a medicinal plant whose calyx is used in Benin to treat anemia. We showed in this study its therapeutic efficacy. For this purpose, a phytochemical screening of the aqueous extract of the calyces of the plant was first carried out to determine the chemical groups. It revealed catechin tannins, galiques tannins, flavonoids, anthocyanins, leucoanthocyanin, quinone derivatives, terpenoids, steroids, reducing compounds, mucilage, anthracene derivatives and free o-glycoside. Some of these compounds include tannins, flavonoids and anthocyanins were also detected in the methanolic extract of this organ of the plant (Obouayeba et al. 2015). The majority of these compounds were detected in leaves of *Sorghum caudatum* also used to treat anemia (Agbangnan et al. 2012).

We then induce hemolytic anemia by injecting phenylhydrazine to *Wistar* rats (Naughton et al, 1995). Anemic rats were treated for two weeks either with distilled water (positive control group) or with vitafer an anti-anemia drug (reference group) or with aqueous extract of *Hibiscus sabdariffa* at 200 or 300 mg / kg body weight (test groups). The biological effect of the different treatments was measured by the change in hemoglobin, the red blood cell count,

erythrocyte constants (mean corpuscular volume, mean corpuscular hemoglobin concentration) and osmotic resistance of red blood cells.

The hemoglobin level is the indicator of anemia. It collapsed at Day 2 in all groups injected with phenylhydrazine. This decrease was corrected in seven days by the vitafer or the extract at 300 mg/kg and in ten days with the extract at 200 mg/kg. Such dose-dependent hemoglobin correction was also observed with the aqueous extract of *Sorghum bicolor* (Sènou et al. 2016). Anemia correction was also observed with the aqueous extract of *Hibiscus sabdariffa* seeds in a hemorrhagic anemia model, in iron deficiency anemia model (Ahmed et al. 2013) or with the plant calyx aqueous extract in an infection anemia model (Umar et al. 2009).

Hemoglobin is a pigment of the Red Blood Cells (RBCs). As hemoglobin, the number of red blood cells collapsed at Day 2 following the hemolysis induced by phenylhydrazine. This decrease was corrected at day 7 by vitafer or extracts. Such results were obtained with aqueous extracts of seeds of *Hibiscus sabdariffa* (Ahmed et al. 2013) and with leaves of the same plant in a model of non-anemic rats (Aba et al, 2016). On D15, the number of red blood cells significantly exceeds the value of D0 only with the extract at 300 mg / Kg. Such erythropoietic potential superior even to that of the reference drug (vitafer) was also observed with the Siddha drug PithaPaandu (Parthibhan et al. 2015).

Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) are constants that also allowed assessing the erythropoietic effect of the extract. The phenylhydrazine first declined MCV at Day 2 causing microcytosis. This decrease was quickly corrected by the extract at day 7 with even a release of macrocytes which in turn has been corrected from day 10.

MCHC moved inversely with respect to the VGM. It significantly down on D7 in treated groups with vitafer or extract at 200 mg / kg indicating hypochromia that was corrected after day 10. The evolution of the MCV and MCHC reflects a more differentiated erythrocytes release towards the end of the experimental period. These results differ from those observed with aqueous extracts of *Cocos nucifera* roots or Sorghum bicolor leaves whose erythropoietic stimulation continued a release of macrocytes even at the end of the experimental period (Sènou et al. 2016, Tchogou et al. 2016). By cons, the erythropoietic mechanism of the extract appeared similar to that observed with the aqueous leaf extract of *Hibiscus cannabinus* that corrected macrocytosis together with anemia (Agbor et al. 2005).

To further investigate this hypothesis, we tested the osmotic resistance of red blood cells in the different groups.

The osmotic resistance is proportional to the rate of young red blood cells circulating (Gbenou et al. 2006). It increased very quickly in the anemic groups, peaked at D7 with higher amplitude for the treated groups, and then gradually declined, suggesting an abundant production of immature red blood cells in early correction phase of anemia.

Finally, we verified the specificity of the extract action by following the evolution of the blood platelets number. Indeed, the number of platelets was not significantly changed by the treatment, indicating a lack of thrombocyte lineage stimulation. This result suggested that the extract did not stimulate all hematopoietic lineages and therefore showed some specificity of action on erythroid. The same observation was done with aqueous root extract of *Cocos nucifera* (Tchogou et al. 2016).

CONCLUSION

This study demonstrated the therapeutic efficacy of aqueous extract of *Hibiscus sabdariffa* calyx in induced hemolytic anemia the treatment. It stimulated erythropoiesis in a dose-dependent manner, corrected anemia after a week and released into the blood well differentiated red blood cells in the second week. It did not affect thrombopoiesis and its action mechanism remains to be elucidated.

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