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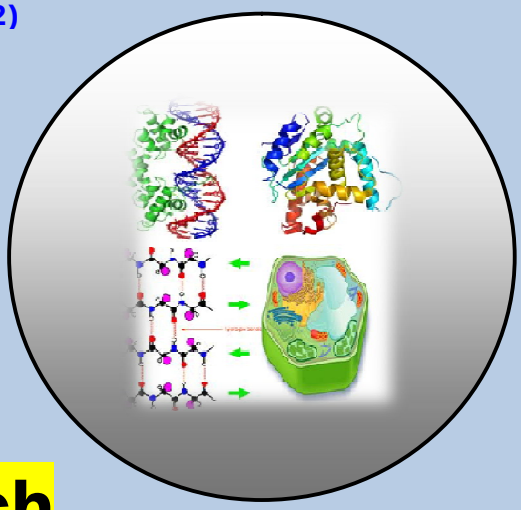

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RESEARCH PAPER

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The *in vitro* Antisickling Properties of Hemodya

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ABSTRACT

Sickle cell disease is a genetic disorder which affects the red blood cells (RBCs). Its therapeutic treatment is complex and very expensive. The patients often turn to traditional medicine to management it. "Hémodya" is a phytomedicine usually used to ameliorating effect of this disease. The phytochemical and Antisickling celled-effect properties of "Hémodya" are being investigated in this study. Qualitative phytochemical tests were used to determine "Hémodya" composition. Antisickling celled-effect properties study carried out on samples of blood taken from 16 informed voluntary sickle cell patients. The sickling of red blood cells (RBCs) was induced using sodium métabisulfite (MBS) 4% followed by treatment with "Hémodya" at different concentrations. The reversibility of the acquired RBCs was study by treatment of SS positive blood with "Hémodya". The potential of inhibition of heamoglobin polymerization and lactate dehydrogenase (LDH) serum activity were determined by the kinetic method, then the Fe^{2+}/Fe^{3+} ratio of patient's heamoglobin by the colorimetric method. Phytochemical test showed the presence of phenolic compounds like gallic and catechic tannins, flavons, leucoantocyans and coumarines. "Hémodya" content also quinones, anthranols, alcaloides, cardiotonic heterosides, fat acids, sterols and terpens. Sodium métabisulfite increase the sickling of RBCs from 22.75±2 to 87.61±2% during 3h. Treatment of sickling cells with "Hémodya" at different concentrations showed that a decrease of the percentage of sickling cells was found in both induced and non induced sickling cells with MBS. The determination of the antisickcling effects of these extracts was directed towards the inhibition of sickle cells polymérisation and the improvement of the Fe^{2+}/Fe^{3+} ratio of HbSS in the presence of "Hémodya". "Hémodya" reduced LDH activity in serum for sickle cell disease patients. "Hémodya" contains bioactive molecules, inhibits and ensures the reversibility of the sickle cells.
Key words: Sickle cell disease, "Hémodya" heamoglobin polymerization inhibition, Fe^{2+}/Fe^{3+} ratio, LDH inhibition, and Phytochemical composition.

INTRODUCTION

The practice of traditional medicine using medicinal plants has a long history in many cultures. This type of health care can be described as herbalism or botanical medicine (Okpuzor et *al*., 2008). The growing sophistication in lifestyles among world populations makes it imperative to refer to herbal practice as alternative or complementary medicine, to appeal to a cross section of people irrespective of their cultural affiliation (Okpuzor et *al*., 2008). This type of herbal practice is gaining increasing attention (Okpuzor et *al*., 2008). Two-thirds of the world's population (mainly in developing countries) relies entirely on such traditional medical therapies as their primary form of health care (WHO, 2004). The use of traditional medicine is important for the treatment and management of a number of diseases in the African continent (Elujoba et *al*., 2005), as a lack of basic health care and medical personnel make it difficult to treat rural populations.

Sickle cell disease (SCD) is a genetic disease which affects the red blood cell (RBC) (OMS 2006). On the biochemical and molecular level, sickle cell disease (SCD) is caused by a point mutation in the ß-globin gene of red blood cells (RBCs) heamoglobin. As result of this mutation, valine (a non-polar amino acid) is inserted into the ß-globin chain at the sixth position in place of glutamic acid (an electrically charged amino acid). The mutation in HbS causes the RBCs containing them to become stiff and sometimes sickle-shaped when they release their load of oxygen. The sickle cell mutation produces a "sticky" patch on the surface of the β-chains when they are not complexed with oxygen (Arnal et Girot, 2002). Because other molecules of sickle cell hemoglobin also develop the sticky patch, they adhere to each other and polymerize into long fibres that cause the deformation of the normal disc biconcave RBC into a sickle shape. Small blood vessels are blocked by the clumping of sickled RBCs, preventing blood supply to various organs (OMS, 2006).

The pathological state appears at the individual homozygote and is characterized by a hemolytic anemia intersected with vaso-occlusive crises which are at the origin of the principal causes of death of sicklemians (Latoundji et *al*., 1991, Bunn, 1997). The prevalence of (SCD) is 2% an average in Africa with a life expectancy lower than 20 years (Gbadoé et *al*., 2001). In the other continents this prevalence is 0.02% (Galactéros 2000).

In Africa, less than 50% of sickle cell patients attain the age of 5 years and less than 18% arrive at the adulthood (Arnal and Girot, 2002). Faced with this pathological epidemiology and troubling mortality, the WHO recognizes this disease like a major public health problem in many countries. Moreover, the economic incidence of the sickle cell disease is of great importance in developing countries. This is why its treatment is out of reach of the populations of the sub-saharan Africa where the use of medicinal plants constitutes part of their culture and tradition. Indeed the African pharmacopeia, which is one of richest of the world and whose development is encouraged by WHO should be, from this point of view, valorized. Thus, the recourse to the local techniques used by traditional healers proves to be necessary. It is accordingly that Etamé in 1980 seriously engaged himself in phytotherapy research in Cameroon to fight against this disease. After almost 20 years of investigation, he identified in the Central Region of Cameroon the medicinal plants with high potentials for the treatment of sickle cell disease.

From this discovery, a drug called "Hémodya" was developed, obtained by decoction on the basis of the barks of 3 plants, and conditioned in the form of syrup. This syrup has been consumed for several years by the sickle cell patients. It results that the product invigorates the muscular system, oxygenates blood and supports the multiplication of RBCs (Etamé, 2000).

According to testimony's of many patients, "Hémodya" reduces the frequency of thier crises and improves their general state. It reduces the rate of hemoglobin S to the profit of the synthesis of A2 and F heamoglobins (Ngogang et *al*., 2003). The oral administration of "Hémodya" does not induce any toxics acute and subacute effects (Kotué et *al*., 2013). However, no studies have yet been carried out to investigate the antisickling properties of «Hémodya». The present study was performed with the aim to investigate the phytochemical composition, antisickling and act mechanisms of this phytodrug.

MATERIAL AND METHODS

"Hémodya", a decoction in syrup form containing 3 medicinal plants: *Cassia siamea Lam (Euphorbiaceae)*, *Delonix regia le Flamboyant (Caesalpiniaceae)*, *Garcinia cowa Rox (Guttiferae)* was provided by the "Pr. ETAME foundation" , P.O Box 14709 Yaoundé-Cameroon, Phone: +237 77765214/99275746, Email: etamewane@yahoo.com. The bottle contents were poured in conical flasks and were dehydrated to powder in oven at 45°C. Qualitative phytochemical screening tests were carried out by the established methods of Ciulei (1982) to determine "Hémodya" composition.

Collection of blood samples and preparation of serum samples

Sickle cell blood (HbSS) samples from 10 males and 6 females aged between 17 to 31 years were obtained from Central Hospital of Yaoundé-Cameroon. The blood samples were collected from confirmed sickle cell patients attending a weekly hematology outpatient clinic at the hospital. The permission of Cameroon National Bio-ethics Committee with authorization N° 184/CNE/SE/2011 was obtained prior to the commencement of this work. Consent from all blood donors were obtained after they were adequately informed of the research objective. The venipuncture method was used for blood collection. At the first time, Blood (4.5 ml) was drawn from each sickle cell patient at steady state using new syringes and needles, spirit swaps and a tourniquet. The blood samples were collected into EDTA tubes for antisickling activity tests of "Hémodya". At the second time, the same procedure was used to collect blood (3ml) to the same patients: 1.5 ml into EDTA tubes for the determination of sickle cell heamoglobin polymérisation experiment and Fe^{2+}/Fe^{3+} ratio. Then, erythrocytes were isolate from 1.2 ml blood samples by centrifugation at a gravitational force of 1500 x g for 15 minutes. Following careful siphoning of the serum with Pasteur pipette, the erythrocytes were by repeated inversion suspended in a volume of isotonic saline (0.9% NaCl) equivalent to the siphoned serum. The erythrocyte suspension was freeze thawed at 4 °C to produce a hemolyzate for the heamoglobin polymerization experiment. Another 1.5 ml blood samples taken in dry tubes were centrifuged at 1500xg for 15 minutes to obtain serum for lactate dehydrogenase (LDH) activity experiment.

Antisickling activity

***In vitro* induction of sickling**

100 µL of SS blood cell suspensions were mixed with 100 µL the presence of 4% sodium metabisulfite solution ($Na_2S_2O_5$) and incubated at 37°C. The time course of the sickling of SS erythrocytes was analyzed microscopically according to the method described by Joppa et al. (2008). The number of cell was counted every one hour after take 10 µL of the mixture diluted 200 times use Marcano liquid. The number of cell was counted every one hour and the percentage of sickling cells was calculated using the formula: (%) Sickling = Number of sickling cells × 100/total cells until obtain the maximum percentage sickling.

***In vitro* anti-sickling activity of "Hémodya"**

A serial of different concentrations of "Hémodya" were prepared in the saline solution. For the assay 500 µL of SS-RBC pre-incubated with 4% $Na_2S_2O_5$ was added to 500 µl of solution of different "Hémodya" for final concentration of 1.37mg/mL (efficacy concentration), 2.74 and 5.48 mg/mL. Each mixture was incubated at 37°C for 3 h (time necessary to obtain maximun sickling). After incubation, 10 µL of the mixture was diluted 200 times use Marcano liquid. 10 µL of each sample was examined under the light microscope and both sickled cells and total cells were counted from five different fields of view across the slide. For the negative control, the solution containing the extract was replaced by the saline solution. The percentage of sickling was calculated using the formula: % of sickling = number of sickling cells × 100/total cells.

For the reversibility assay, freshly collected HbSS blood was diluted in 1:1 ratio with 0.9% normal saline (negative control) or test solution containing different "Hémodya" for final concentration of 1.37 (efficacy concentration) and 2.74 mg/mL. The experiment was followed as mentioned above and the percentage of sickling cells was calculated.

Determination of sickle cell polymerization inhibition

Sickle cell heamoglobin polymerization experiment or HbSS polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm using 2% Sodium metabisulphite as reductant or deoxygenating agent (Nwaoguikpe and Ejele, 2010). 4.4 ml of 2% solution of sodium metabisulphite ($Na_2S_2O_5$), 0.5 ml normal saline and 0.1 ml of antisickling agent were pipetted into a cuvette shaken and the absorbance read at 700 nm every two minutes for 30 minutes. This represented the control. Distilled water was used as blank in all assays. In the main assay, 4.4 ml of 2% solution of sodium metabisulphite, 0.5 ml of "Hémodya" (1.37mg/ml) and 0.1 ml of hemoglobin solution (HbSS) were pipetted into a cuvette and the optical density reading taken as above. The rates of heamoglobin polymerization were calculated from the formula of average change in optical density/absorbance against time in minutes.

$$RP = \sum. \frac{DOf - DOi}{t}$$

RP = rate of polymerization, ODf =final absorbance at time t, t = time of assay in minutes, ODi = initial absorbance / optical density at zero time.

The percentage cell blood polymerization inhibition were calculated from the formula

$$PPI\ essay\ (\%) = \frac{RP\ essay\ x\ 100}{RP\ control}$$

Determination of Fe^{2+}/Fe^{3+} ratio of sickle cell blood

The effect of "Hémodya" on the Fe^{2+}/Fe^{3+} ratio in blood were performed as previously described by Davidson and Henri (1974). An aliquot of sickle cell blood (20 µl) was added to distilled water (5.0 ml) and normal saline (20 µl) to serve as a control. The standard and test solutions contained the same reagents as the control except that an aliquot (20 µl) of "Hémodya" (1.37mg/ml) was added instead of normal saline. Samples were incubated for 60 min and the absorbance measured by spectrophotometer at wavelengths of 540 nm and 630 nm to determine the percent oxyheamoglobin and metheamoglobin respectively. The ratio of oxyheamoglobin and metheamoglobin was calculated by dividing the percentage oxyheamoglobin by that of metheamoglobin.

$$R = Fe^{2+} / Fe^{3+}$$

Determination of lacta I) activity

It was made according to the method described by Afolabi and al. 2012 performed according to the manufacturer's instructions. An aliquot (20 µl) of the "Hémodya" (1.37mg/ml) was dispensed into a cuvette containing the kit reagent (1.0 ml), and serum samples (40 µl) of the HbSS patients. Serum samples from healthy contributors were used for the control. All the reactions were carried out at 25°C. The initial absorbance at 340 nm was taken within 30 s, and the absorbance changes were taken afterwards at every 1 min for 3 min. The LDH activity was calculated using the formula below as provided by the LDH kit manufacturer.

$$LDH\ \left(\frac{UI}{L}\right) = 8095\ x\ \sum . \frac{DOf - DOi}{t}$$

Statistical Analysis

The results were expressed as mean ± standard deviation. Data were analyzed using analysis of variance (ANOVA) of Kruskall-Wallis with the software Sigma Start version 3.01A analysis software. Treatments with a "P" value of < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The qualitative screening phytochemical of "Hémodya" showed the presence of phenolic compounds like gallic and catechic tannins, flavons, leucoantocyans and coumarines. "Hémodya" content also quinones, anthranols, alkaloids, cardiotonic heterosides, fat acids sterols and terpens. These phytochemical constituents seemed to have the potential to act as a source of useful drugs and also to improve the health status of consumers. In fact flavonoids (Middleton and Kardasnami, 1993), tanins (Harborne, 1998), sterols (Beutchet, 1997) have anti-inflammatory properties.These properties can be made profitable in the assumption of responsibility of the ostéoarticulars painful crisis, the most usual expression met at the sickle cells patients.

Among identified molecules, many have antibactériens and antiviral effects. They are alkaloids (Harborne, 1998), flavonoids (Middleton and Kardasnami, 1993), glycosides (Materska and *al*., 2003), sterols (Beutchet, 1997). Such compounds can help the sickle cells patients to fight against the severe infections which are the principal cause of the deaths.

In oder to verify "Hémodya" antisickling activity, an *in vitro* bioassay has been performed. The result of induction of sickling with sodium métabisulfite (4%) shows an increase in sickling from 22.75±2 to 87.61±2% after 3h of induction may be a average rate of increase of 74.03±2% (fig 1). Figure 2 illustrate the various morphological states of red blood cells observed under the optical microscope during various hours until obtaining a maximum falciformation. At time t = 0h, we noted a prevalence of normal red blood cells. About 3h, the sickling cells become majority. In this study, the percentage of the falciformation obtained after 3h of incubation is lower than 96.5% found by Joppa et al. 2008 at the same period. It is higher than 80% and 52.08% values obtained respectively by Elekwa et *al*. (2005) after 1h, then Nanfack et *al*. (2013) at 2h of incubation. Thus sodium metabisulfite creates hypoxic conditions for red blood cells leading to the loss of the morphology and sickled erythrocytes. In vitro deoxygenating of RBC by sodium metabisulfite caused progressive aggregation and polymerization of the individual hemoglobin molecules (Steinberg, 1993, Glactéros, 2001). The process of gelation (polymerization) of hemoglobin molecules increases the formation of sickling cells. The sickle cell hemoglobin (HbS) is a product of a defective genetic code of heamoglobin molecule.

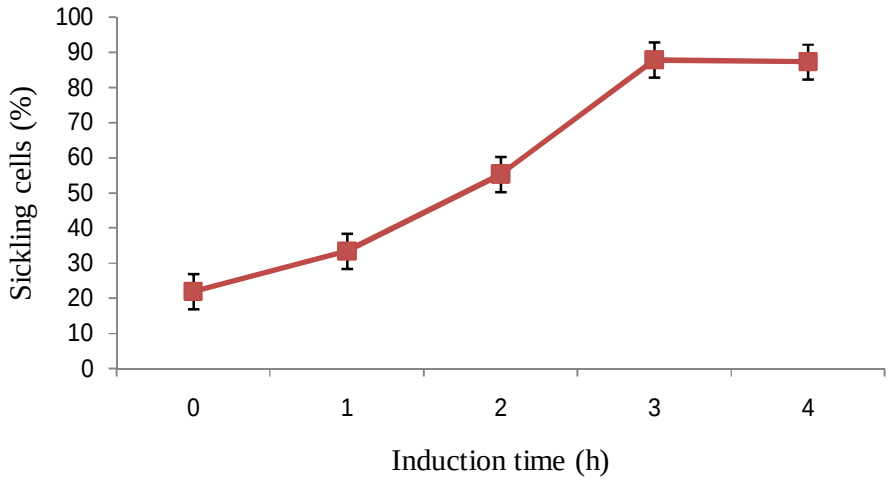

Figure 1. Percentage of falciformation according to time after induction of sickling.

After treatment of red blood cells with "Hémodya" at different concentrations, decrease of the percentage of sickling cells was observed. This percentage of sickling varied from 24.5±5 at 1.37 mg/ml, 23.34±5 at 2.74 mg/ml and from 23.14±5% at 5.48 mg/ml (fig 3) may be a falciformation average rate of inhibition of 71.36±7%, 72.41±7% and 72.51±7% respectively. These results are shown that inhibition undepending on the "Hémodya" dose although was no significant (p>0.05).

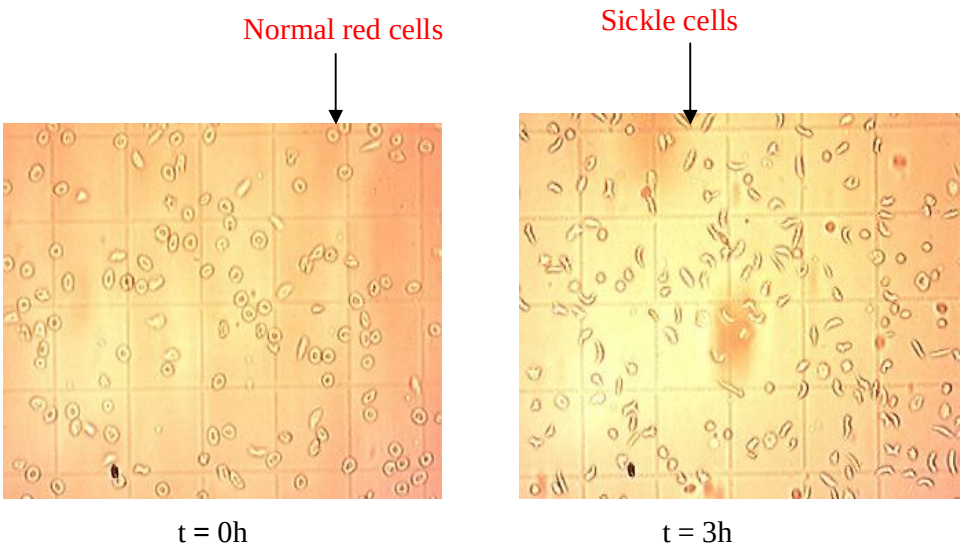

Figure 2. Morphological states of patients red blood cells observed under the optical microscope (40x/0.65) according to time.

These rates of inhibition are lower than of 81% obtained by Joppa et al. (2008) with the extract of the sheets of *M. lucida* and are significant with the sight of those obtained by other authors. Indeed, Sofowora et *al*. (1975) obtained with the extract of the roots of *Fagara xanthoxyloïdes* an inhibition of falciformation of 53%, Lyamu (2002) reached a value of 50% of the rate of inhibition with NIPRISAN, a antisickling product containing 4 medicinal plants (seeds of *Piper guineense*, fruits *of Eugenia caryophyllum*, stems of *Pterocarpus osun* and sheets of *Sorghum bicolor*). Also, Nanfack et *al.* (2013) has obtained 39.5% of inhibition with the extract of the fruits of *Xanthoxylum heitzii*.

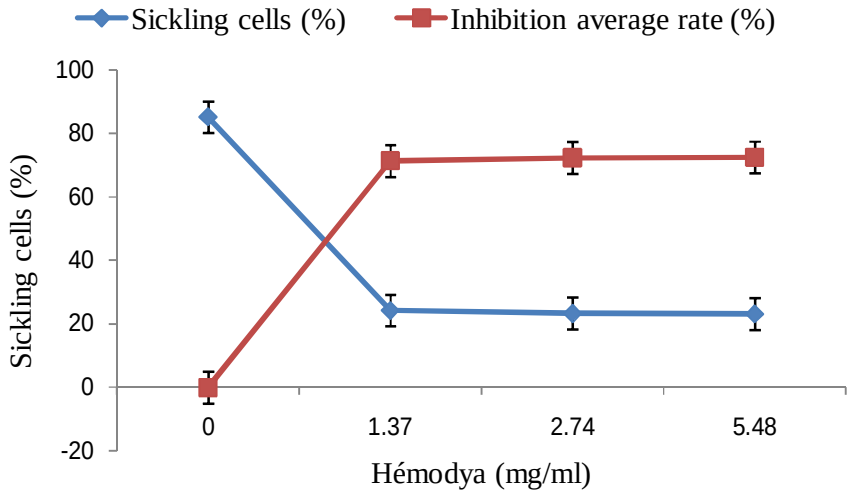

Figure 3. Evolution of percentage of falciformation of sickling cells and its average rate after treatment by the increasing concentration of "Hémodya" with sodium métabisulfite (4%).

The reversibility of the sickling cells was noted through inhibition after 3h of SS-RBCs incubation with "Hémodya" at 1.37 and 2.74mg/ml. The average rate of this reversibility varied between 66.14±4 (1.37 ml/mg) and 64.47±6 (2.74mg/ml) although was no significant ($p>0.05$) and no dose dependent for 'Hémodya" (fig 4 and 5). These rates of reversibility are higher than the value of 39% obtained by Nanfack et *al*. (2013) with the extract of the fruits of *Xanthoxylum heitzii*. The activity of "Hémodya" could be due to the presence of some bioactive compounds they possess. In fact the antisickling activity could be linked to their ability either to inhibit in vitro polymerization of hemoglobin or to some structural modification linked to the environment of hemoglobin by "Hémodya". Several researches have established that the capability of phenolic compounds to avoid in vitro polymerization would act while being intercalated between the hydrophobic connections, thus reducing cohesion between polymers of heamoglobin (Nwaoguike and Uwakwe 2005). This antisickling activity would be also allotted to anthocyanes (Mpiana et *al*., 2008).

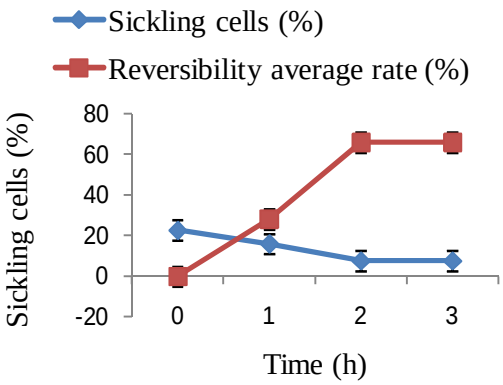

Figure 4. Evolution of percentage of falciformation of sickling cells according the time after treatment with "Hémodya" at 1.37mg/ml and in absence of sodium métabisulfite 4%.

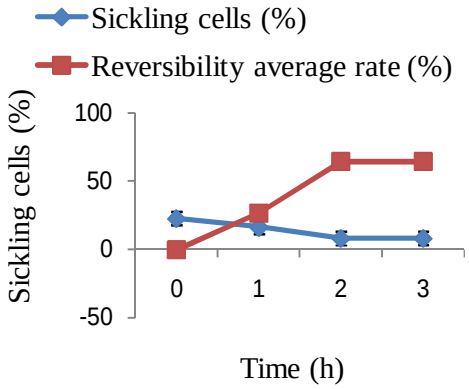

Figure 5. Evolution of percentage of falciformation of sickling cells according the time after treatment with "Hémodya" at 2.74mg/ml and in absence of sodium métabisulfite 4%.

Regarding the mechanism of action, we were investigated to ascertain the ability of "Hémodya" to inhibit polymerization of sickle cell heamoglobin (HbS), improve the Fe^{2+}/Fe^{3+} ratio and lower the activity of lactate dehydrogenase (LDH) in blood plasma.

Figure 6 shows the ratios variation of polymerization of HbS control (without "Hémodya") and "Hémodya" treatment studied according to time. From there two ratios variation of polymerization, the potential of inhibition polymerization (PIP) of HbS by "Hémodya" is estimated to 62.3±14%. This inhibition is the resultant of the action of "Hémodya" on the intensity of the turbidity (proportional to the optical density) which is in connection with the polymerization caused by the sodium métabisulfite (2%). This value is lower than 92% obtained by Ojiako et *al*. (2012) with the rough extract of *Cucurbita pepo* grains. It is also lower than 80.86% and 77.93% values obtained respectively by Nwaoguikpe and Ejele (2010) with the aqueous extract of sheets and freezing of aloe vera (*aloe barbadensis*), lower than 95% but higher than 50% values obtained respectively by Nwaoguikpe and Uwakwe (2005) with the aqueous extracts of dried prawn (*Astacus red*) and dried fish (*Tilapia*).

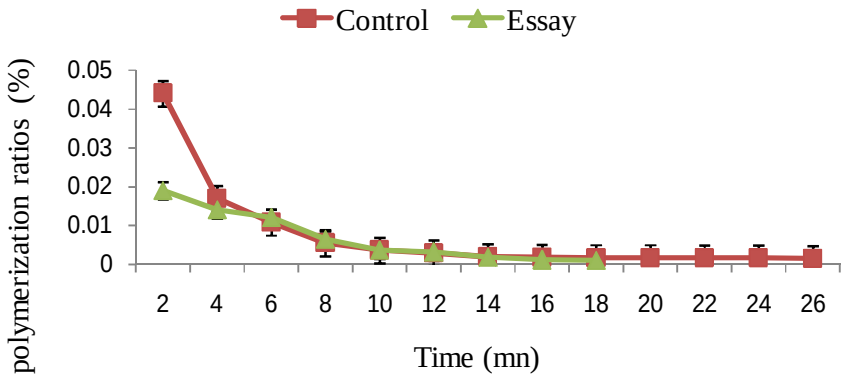

Figure 6. Ratios variation of polymerization of HbS control and "Hémodya" treatment study according to time.

Fe^{2+}/Fe^{3+} ratios in HbS control were significantly reduced ($P<0.05$) compared with "Hémodya" treatment studied Figure 7). Indeed, these ratios were estimated to 6.67±2% for control and 10.98±4% for the essay with an average rate of increase to 35.24±16%.

This value is lower than 99.53% obtained by Ojiako et *al*. (2012) with the rough extract of *Cucurbita pepo* grains. It is also lower than 78% and 46.98% values obtained respectively by Nwaoguikpe and Ejele (2010) with the aqueous extract of the sheets and the freezing of aloe vera (*Aloe barbadensis*), lower than 49,23% but higher than 6.09% values obtained respectively by Nwaoguikpe and Uwakwe (2005) with the aqueous extracts of dried prawn (*Astacus red*) and dried fish (*Tilapia*). In all the cases substitution in the chain β of the glutamic acid in position 6 by valin involves the precipitation of heamoglobin in the red blood with falciformation supporting the oxidation of the ferro-iron (Fe^{2+}) to ferri-iron (Fe^{3+}) which cannot collect O_2 (anorexia) any more (Dahmani et *al*., 2009). "Hémodya" with chelating activity would prevent the oxidation of the ferro-iron and increase Fe^{2+}/ Fe^{3+} ratio. It thus improves affinity of heamoglobin with oxygen.

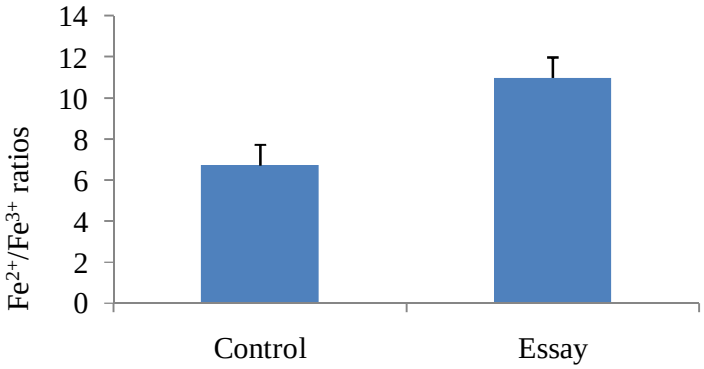

Figure 7. Effect of "Hémodya" treatment studied on the Fe^{2+}/Fe^{3+} ratios of HbS compared to the control.

Figure 8 shows a significant difference between the activities of lactate dehydrogenase (LDH) of the serum sickling cells patients of the "Hémodya" treatment studied compared with control. Indeed, this activity is estimated to 482.31±104 UI/L for the control and 210.67±42 UI/L for the essay with an average inhibition of the activity to 55.37±16%. This value is lower than 96.97% obtained by Afolabi et *al*. (2012) with the methanolic extract of the of *Solenostemon monostachyus* sheets. It higher than 40% and 12% obtained respectively by Nwaoguikpe and Uwakwe (2005) with the aqueous extracts of dried prawn (*Astacus red*) and dried fish (*Tilapia*), and with of 25.4% obtained by Afolabi et *al*. (2012) with the oil of *Carica papaya* seeds. Indeed the LDH allows a reversible reduction of the pyruvic acid in lactic acid. When oxygenation is insufficient at the sickle cells patients, pyruvate is reduced in lactic acid which acidifies the medium, thus causes sickle cells hémolysis (Hamadah et al. 2010).The inhibition of enzyme (LDH) by "Hémodya" gives the possibility of decreasing the production of lactate. This situation stabilizes the sickles cells thus reducing to their destruction with a significant improvement of anemia among sickle cells patients, and a better red blood oxygenation.

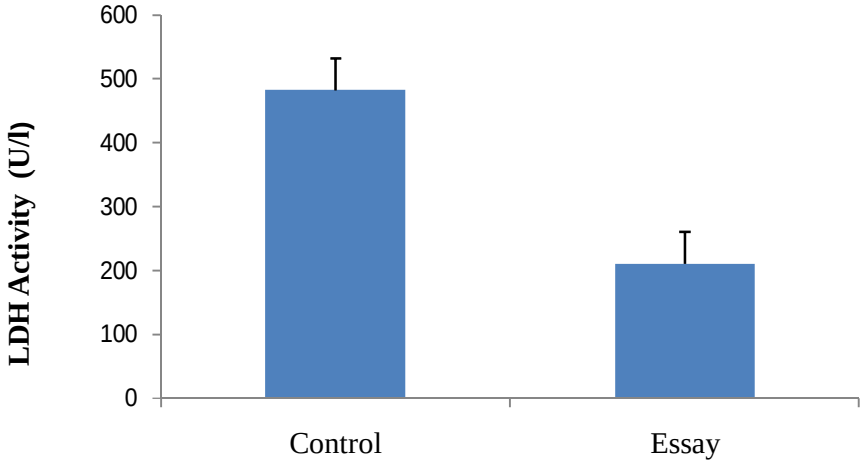

Figure 8. Effect of the "Hémodya" on the LDH activity of serum of sickling cells patients compared to the control.

CONCLUSION

"Hémodya" has an *in vitro* antisickling effect. The antisickling effect of "Hémodya" were investigated shows the ability to inhibit polymerization of sickle cell heamoglobin (HbS), improve the Fe^{2+}/Fe^{3+} ratio and lower the activity of lactate dehydrogenase (LDH) in blood plasma. These results prove to be effective in the management of sickle cell disease (SCD). "Hémodya" isolated compounds and a clinical trial to investigate the antisickling effect *in vivo* warrant our further studies.

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