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### HPLC profiling, *in vitro* antisickling and antioxidant activities of phenolic compound extracts from black bean seeds (*Phaseolus vulgaris* L.) used in the management of sickle cell disease in the West Region of Cameroon

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#### ABSTRACT

Natural molecules from food have been used to manage sickle cell crises. As a genetic blood disorder, treatment is complex and expensive. This study was carried out to establish the phenolic compounds profile of black bean seeds (*Phaseolus vulgaris* L.) commonly used by some families in the Western Region of Cameroon to manage sickle cell disease and to evaluate their *in vitro* antisickling, membrane stability and antioxidant properties. Free, bound and total phenolic contents were estimated to be 0.1±0; 0.108±0 and 0.212±0 mg EAG/g of sample respectively. Free phenolic compounds contain ferulic acid (0.013 µg/g), while bound phenolic compounds contain gallic acid (2.13 µg/g) and ferulic acid (0.037 µg/g). Free phenolic compounds had the higher rates of inhibition (82.26±2%), reversibility (69.86±3%) of sickling and the best effect on membrane stability of erythrocytes. Phenolic extracts from black bean seeds also showed a high global antioxidant activity with free phenolic compounds (28.42± 0 mgFell/100g). Total phenolic compounds showed a better activity on DPPH radical with a IC50 of 2.42±1µg/µL while free phenolic compounds showed a better activity on scavenging hydroxyl radical with a IC50 of 1.5±0.5µg/µL. These results may justify the use of black bean seeds by sickle cell patients from Cameroon.

**Keywords:** Antioxidant, antisickling, black bean seeds, HPLC, phenolic compounds, sickle cell disease.

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## Introduction

Several varieties of dry beans (*Phaseolus vulgaris* L.) varying in seed shape, size, and color are consumed throughout the world primarily as an important source of plant proteins (Beninger and Hosfield 2003). However, in recent years dry bean phenols have received considerable attention mainly due to their health promoting properties (Beninger and Hosfield 2003; Cardador-Martinez et al. 2002). Preliminary results suggest that phenolic compounds found in beans and other plant products provide protection against certain types of cancers, cardiovascular, and other chronic diseases due to their antioxidant activity and ability to chelate metal catalyst (Beninger and Hosfield, 2003; Heimler, 2005). Phenolic compounds have antisickling effect and are enables to reduce oxidative stress that contributes to sickle cell crisis (Tatum and Chow, 1996). In fact, as a genetic hereditary disease, no specific drugs are available. However several treatments have been investigated (WHO, 2011). Bone marrow transplant is the only sure treatment and available to date but very expensive (Mpiana et al., 2008). Several studies have described the antisickling effect of food extracts and especially their phenolic compounds (Ogoda et al., 2002).

This is the case, for example of phenolic compounds from *FicusSy comorus* seeds (Ramde-Tiendrebeogo et al., 2012). Recent investigation has shown that black bean seeds (*Phaseolus vulgaris* L.) cultivars from Cameroon West Region are used to manage sickle cell disease (SCD) (Kotue et al., 2016). This study was carried out to profile phenolic compounds present in black bean seeds (*Phaseolus vulgaris*. L) that in general used by some families in the Western Region of Cameroon to manage the sickle cell disease and to evaluate their *in vitro* anti-sickling, membrane stability properties and, antioxidant potential.

## Materials and methods

### Collection and preparation of plant materials

The seeds of black beans sample (Figure 1) were obtained from sickle cell patients families and authenticated as PNN, a wild variety at the Agricultural Institute of Research for the Development of Foubot station, Cameroon. At the Department of Biotechnology of the Pondicherry University, India, the seeds (1kg) were weighted (Infra Digi balance), pulverized using an electrical grinding machine (Preethi). The flour material was stored in cold room at 4°C for further analysis.



**Figure 1:** Photograph of black bean seeds (*Phaseolus vulgaris* L.) PNN (wild variety) collected from local sickle cell patient's families of west region of Cameroon

## Free and bound phenolic compounds extraction, phenolic content and HPLC analysis.

Free and bound phenolic compounds were extracted according to the methods described by Chen et al. (2016). Otherwise, total phenolic compounds were obtained by mixing free and bound phenolic compounds after their extraction. Phenolic content (TPC) in each fraction was determined by the Folin-Ciocalteu colorimetric method as described by Ndolo and Beta (2014). The chromatographic separation was carried out on an HPLC (Shimadzu) equipped with a photodiode array detector and was conducted as described by Yu et al. (2015).

### *In vitro* Antisickling activity

All anti-sickling experiments and hemolysis test were performed as previously reported (Joppa et al. 2008; Jaja et al. 2000) using a EDTA suspension of freshly collected blood after receiving an ethical clearance number 00526/CRERSHC/2018 with consent from all blood donors.

### Antioxidant activity of amino acids extract

The Total antioxidant activity by ferric reducing antioxidant power assay (FRAP) was used to

determine the total antioxidant activity which measures the reduction of ferric ion to the ferrous form in the presence of antioxidant compounds according to the method performed by Benzie and Strain (1996). The DPPH free radical scavenging assay was carried out for the evaluation of the antioxidant activity using the method described by (Jain et al. 2008). However, hydroxyl radical scavenging activity was measured according to a previously method proposed by (Yu et al. 2002).

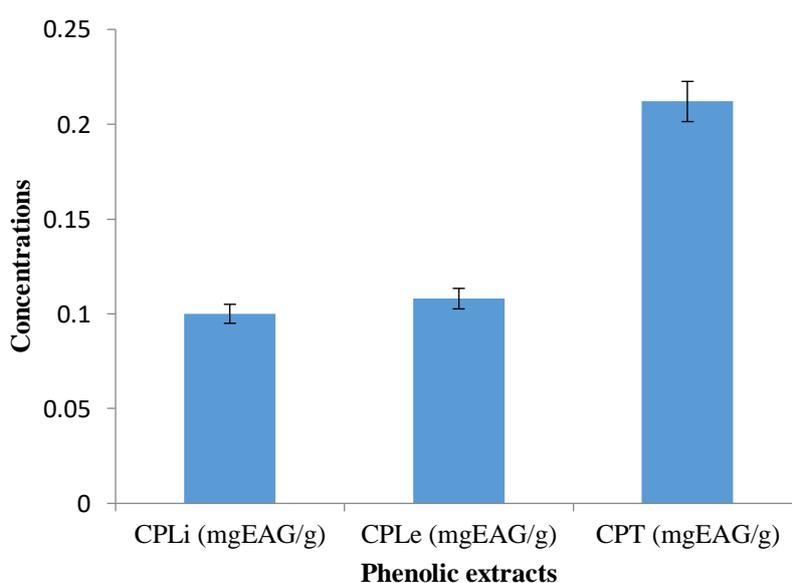
### Statistical analysis

The results were expressed as mean  $\pm$  standard deviation. Data was analyzed using Analysis of Variance (ANOVA) of Kruskal-Wallis with the software Sigma Start version 3.01A analysis software. Statistical data were considered significantly different at 95% confidence interval ( $p < 0.05$ ).

## Results and discussion

### Determination of phenolic content

The phenolic compounds of the black bean seeds can be classified into free and bound forms. Free phenolic compounds, bound phenolic and total phenolic contents were estimated to be  $0.1 \pm 0$ ;  $0.108 \pm 0$  and  $0.212 \pm 0$  mg EAG/g of sample respectively (Figure 2).

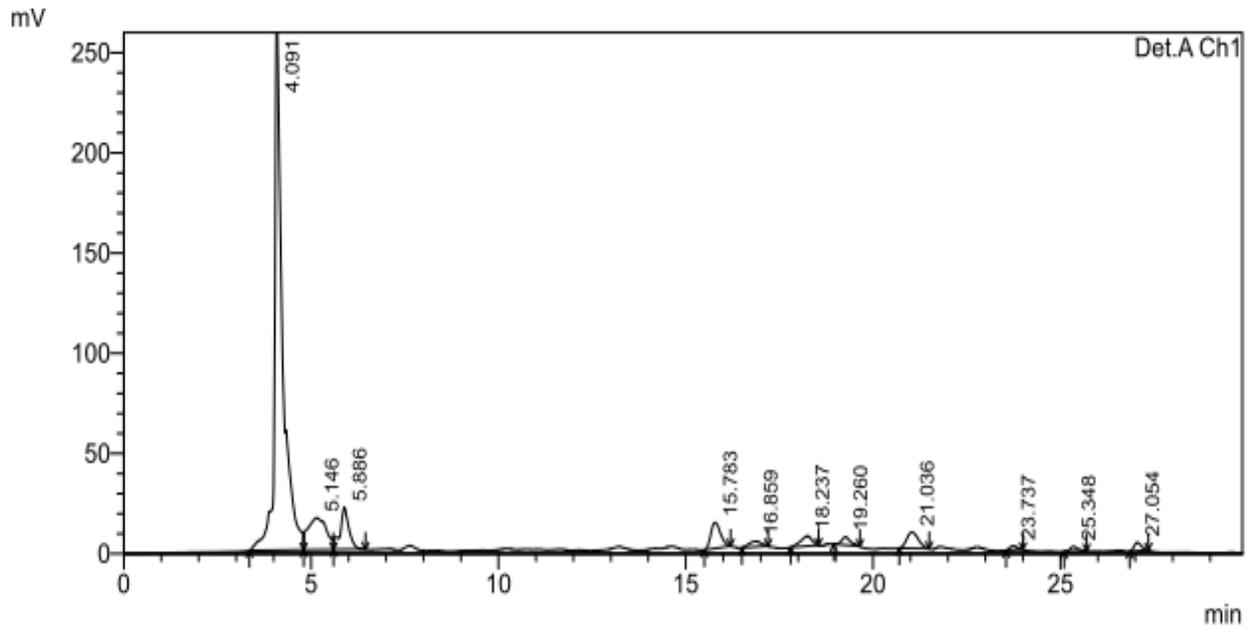


**Figure 2:** Phenolic extracts from black bean seeds (0.1mg EAG/g; 0.108 mg EAG/g and 0.212 mg EAG/g) representing free (CPLi), bound (CPLe) and total phenolic (CPT) extracts respectively.

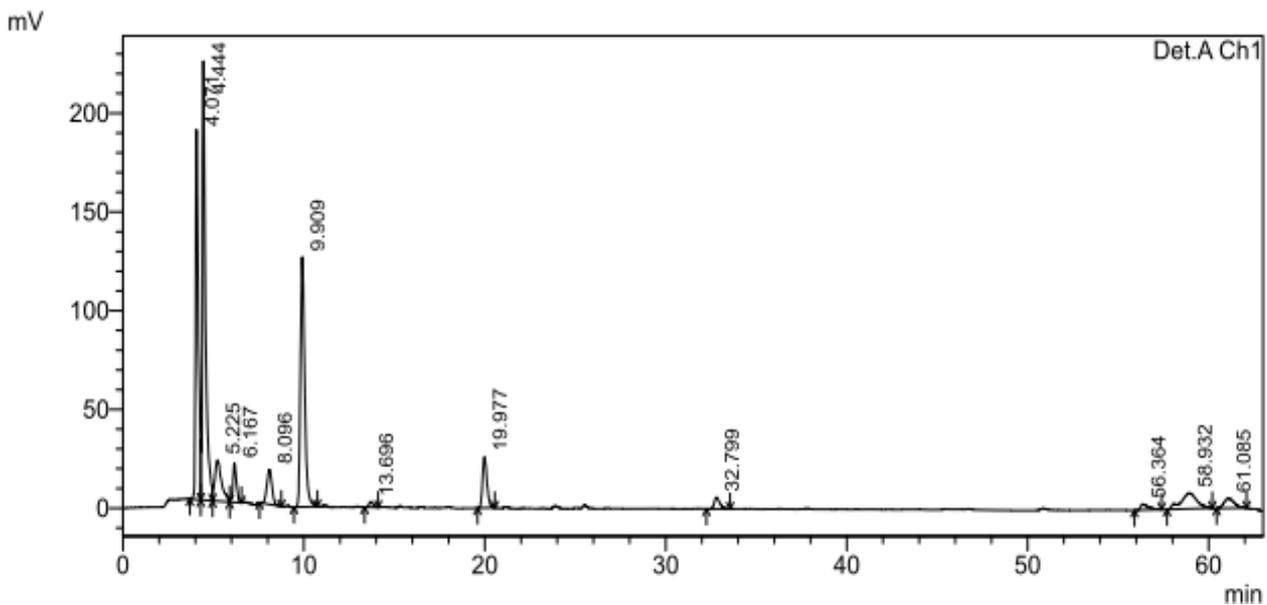
Total phenolic extract (CPT) was low, in agreement to the results reported by **Maria and al. (2016)** which studied other black bean seeds Southern Italian of *Phaseolus vulgaris* L. with values that vary between 0.24 et 0.436 mg EAG/g. In addition, the values of the free phenolic (CPLi) and bound phenolic (CPLe) extracts content were lower than those

obtained by **Fan and Beta (2016)** who found values ranging from 0.79 to 1.2 mg/g and 0.5 to 0.65 mg/g respectively for Three Common Bean Varieties (*Phaseolus vulgaris* L.) from central Malawi.

A total of eleven free (Figure 3) and eleven bound phenolic compounds (Figure 4) were found in black bean seeds.

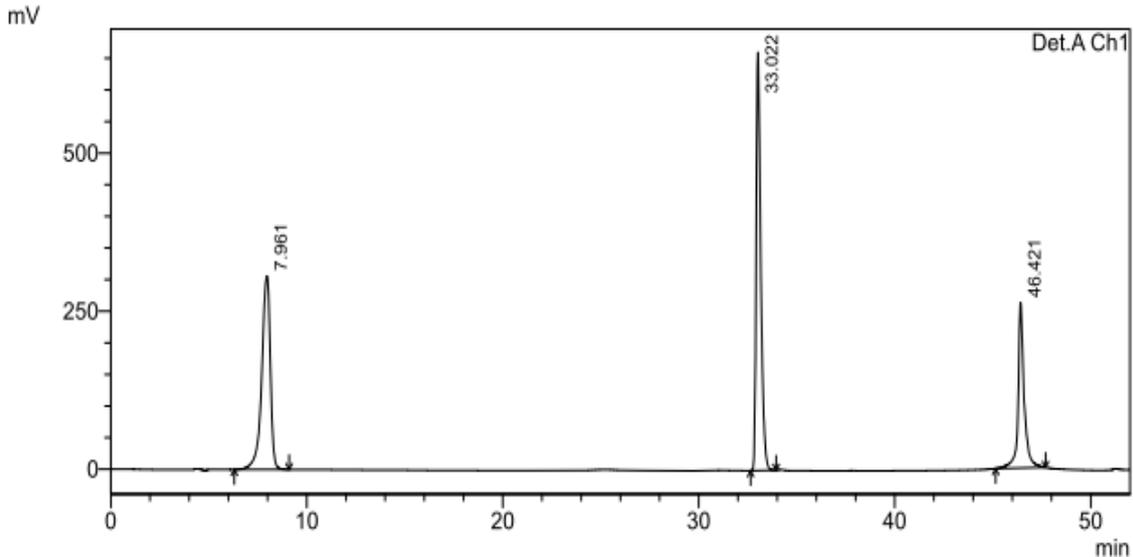


**Figure 3:** HPLC chromatogram of free phenolic compounds from black bean seeds.



**Figure 4:** HPLC chromatogram of bound phenolic compounds from black bean seeds.

To identify some of the compounds a couple of standards were also analysed by HPLC. The standards used were gallic acid, ferulic acid and quercetin. Their retention times were estimate to be 7.967; 33.036 and 46.469 min respectively (Figure 5).



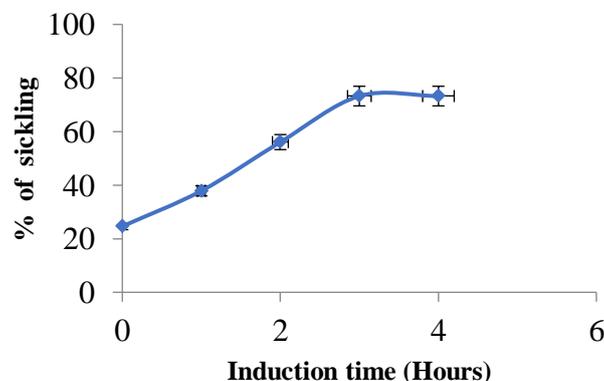
**Figure 5:** HPLC chromatogram for standards: Gallic acid (RT 7.961 min), Ferulic acid (RT 33.022 min) and Quercetin (RT 46.421). HPLC mobile phase: Ethanol/water (95%).

The profiles of free phenolic compounds (Figures 3) upon examination, shown a peak with a retention time near 33.036 min identified it as ferulic acid. Its concentration estimated on the basis on peak area was found to be 0.013  $\mu\text{g/g}$ . Figure 4 showed two peaks with a retention time of 7.961 and 33.036 min which identified one as gallic acid and the other as ferulic acid. Their concentrations were estimated to be 2.13 and 0.037  $\mu\text{g/g}$  respectively. Beans are good source of phenolic compounds. Gallic, chlorogenic and caffeic acid were found to be the major phenolic compounds in black bean Ramirez et al. 2014. Similar trend was observed in a study of quantitation of phenolics from the seeds of two

common beans consumed by Rwandans (Joseph et al. 2014). The observed values are much higher for gallic and ferulic acids compared to the study samples.

#### ***In vitro* Antisickling activity**

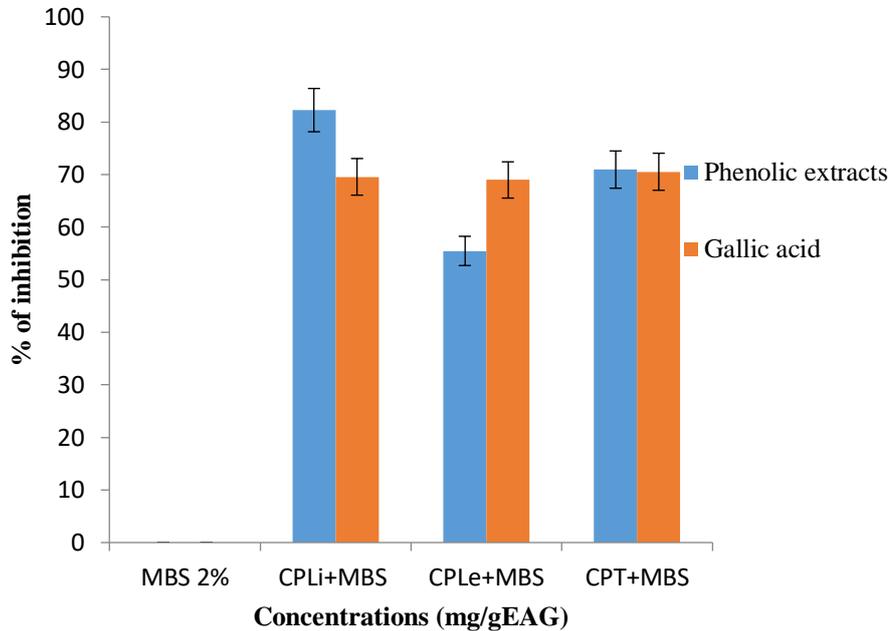
Sodium metabisulfite 2%, added to sicklier red blood cells at equal volumes provoked sickling of red blood cells (Figure 6). At time 0 hour, sickling percentage was 28.4% and after 3 hours of incubation sickling increased on average from  $24.61 \pm 1.3\%$  to  $73.25 \pm 2.4\%$  and remained constant with time. The maximum number of sickling was obtained after 3 hours, suggesting that this is the time necessary to obtain maximum sickling.



**Figure 6:** Percentage of sickling as a function of time after induction of red blood cells with MBS (2%). At time 0 hour, MBS 2% was replaced by NaCl 0.9%.

The Figure 7 show that the rates of inhibition of sickling of red blood cells are significantly ( $P < 0.05$ ) different for all concentrations of phenolic compounds extracts compared to the

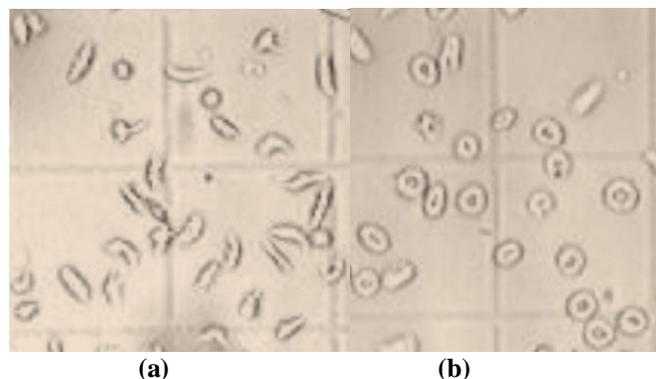
control. These rates were  $82.26 \pm 2\%$ ;  $55.47 \pm 3\%$ , and  $70.94 \pm 1\%$  respectively for free ( $0.025 \mu\text{g/mL}$ ), bound ( $0.027 \mu\text{g/mL}$ ) and total phenolic compounds ( $0.053 \mu\text{g/mL}$ ).



**Figure 7:** Rate of inhibition of sickling of phenolic extracts from black bean seeds (0.1mgEAG/g; 0.108 mgEAG/g and 0.212 mgEAG/g) representing free (CPLi), bound (CPLe) and total phenolic (CPT) extracts respectively.

Figure 8 illustrates the morphology of SS blood erythrocytes (Control) and that of SS blood erythrocytes in the presence of phenolic extracts. (a) shows that nearly maximum of RBCs adopt a sickle-shape in hypoxic conditions which, additionally, confirms the SS

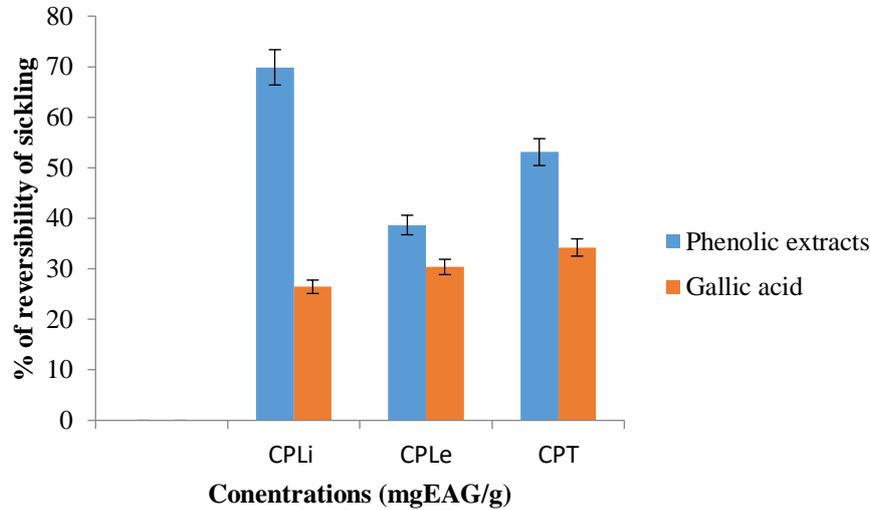
nature of the used MBS. As shown in (b), in the same experimental conditions, these sickle erythrocytes present a different morphology: they almost recover the biconcave normal form. This is unambiguously due to the presence of phenolic compound extracts.



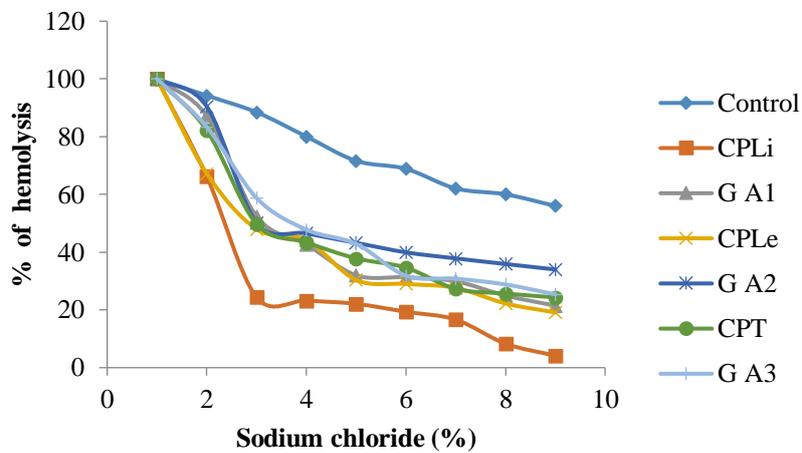
**Figure 8:** Morphological states of patients red blood cells observed under the optical microscope (40x/0.65) according the treatment with free (CPLi) phenolic extracts. (a) with MBS 2%; (b) with MBS 2% + (CPLi) after 3 hours induction.

Otherwise, the rate of reversibility of sickling of red blood cells (Figure 9a) are significantly ( $P < 0.05$ ) different for the same concentrations of phenolic extracts as compared to the control. These rates were  $69.86 \pm 3\%$ ;  $38.66 \pm 2\%$  and

$53.1 \pm 1\%$  respectively. Hemolysis also decreased for different extract concentrations showing the stability effect on the membranes of erythrocytes (Figure 9b).



**Figure 9a:** Reversibility rate of sickling due to phenolic extracts from black bean seeds (0.1mgEAG/g; 0.108 mgEAG/g and 0.212 mgEAG/g) representing free (CPLi), bound (CPLe) and total phenolic (CPT) extracts respectively.



**Figure 9b:** Effects of phenolic extracts from black bean seeds on hemolysis

Gallic acid and ferulic acid were tested *in vitro* and their anti-sickling properties were confirmed. This has created interested to look for their presence in the different phenolic extracts of black bean. HPLC revealed the presence of eleven each of free and bound phenolic compounds in black bean seeds. Gallic acid and ferulic acid served as the

standards and their retention times (RT) in HPLC were 7.967, 33.036 and 46.469 min respectively. The HPLC profile of free phenolic compounds upon examination showed a peak with a RT near 33.036 min indicating its identity as ferulic acid. Its concentration was estimated to be 0.013  $\mu\text{g/g}$ . HPLC of bound form of phenolic compounds showed presence of two

peaks with RT's corresponding to gallic and ferulic acids with their concentrations estimated to be 2.13 and 0.037  $\mu\text{g/g}$  respectively. The antisickling effects of these phenolic extracts thus may be attributed to these two compounds. In fact ferulic acid, galic acid and like many natural phenols were tested *in vitro* and their anti-sickling properties were confirmed (Moody et al. 2003).

Sickle red blood cells are fragile and susceptible to hemolysis. Compared to the control ( $p < 0.05$ ) all phenolic extracts had a positive influence on the stability of erythrocyte

membranes. However, the CPLi (0.025 $\mu\text{g/mL}$ ) showed better membrane stability followed by CPLie (0.027  $\mu\text{g/mL}$ ) and finally CPT (0.053 $\mu\text{g/mL}$ ). These results corroborate those found by Selva et al. (2017) who had shown that phenolic compounds of *Fragaria xananassa* D. had a positive influence on the stability of erythrocyte membranes.

#### Antioxidant activity

Table 1 show the evaluation of the antioxidant properties of amino acids extract using gallic acid as references.

**Table 1:** Evaluation of the reduction and scavenging properties of phenolic extracts using gallic acid as references.

Compounds	Free phenolic compounds	Bound phenolic compounds	Total phenolic compound	Gallic acid
FRAP (mgFeII/100g)	28,42 $\pm$ 0,0 <sup>a</sup>	26,25 $\pm$ 0,6 <sup>b</sup>	6,80 $\pm$ 0,0 <sup>c</sup>	31,3 $\pm$ 0,1 <sup>d</sup>
DPPH (IC <sub>50</sub> mg/mL)	6,46 $\pm$ 1,5 <sup>d</sup>	3,67 $\pm$ 0,0 <sup>e</sup>	2,42 $\pm$ 1,5 <sup>e</sup>	14,68 $\pm$ 2,0 <sup>f</sup>
OH (IC <sub>50</sub> mg/mL)	17,86 $\pm$ 0,0 <sup>g</sup>	1,5 $\pm$ 0,5 <sup>h</sup>	43,16 $\pm$ 1 <sup>i</sup>	85 $\pm$ 5 <sup>j</sup>

\* Mean values from triplicate measurements  $\pm$  standard deviation. Values in the same row followed by different Superscripts are significantly different ( $p < 0.05$ )

Phenolic extracts from black bean seeds also showed a high global antioxidant activity with free phenolic compounds (28.42 $\pm$ 0 mg FeII/100g). This value remains below 33.67 $\pm$ 1.12mgFeII/100g obtained by Kwuimgoin et al. (2017) with phenolic compounds of red kidney beans seeds. Total phenolic compound showed a better activity on DPPH radical with IC<sub>50</sub> of 2.42 $\pm$ 1 $\mu\text{g}/\mu\text{L}$ . This value is lower than IC<sub>50</sub> obtained by Ramde-Tiendrebeogo et al. (2012) with the phenolic compounds of *Ficussycomorus*. (9.60 $\pm$ 0.02 $\mu\text{g}/\text{ml}$ ). Fe<sup>2+</sup> is a powerful pro-oxidant that can react with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to produce hydroxyl radicals ( $^{\circ}\text{OH}$ ), the most aggressive free radical found *in vitro* (Javanmardi et al. 2002, Vassale et al. 2004). Free phenolic compounds showing a better

activity on scavenging hydroxyl radical with a IC<sub>50</sub> of 1.5 $\pm$ 0.5  $\mu\text{g}/\mu\text{L}$ .

**Conclusion:** The phenolic compounds extracted from black bean seeds demonstrated inhibitory and reversibility activities on sickling and a stability effect on the membranes of erythrocytes. They also showed a free radical scavenging activity and an antioxidant potential. These results may justify the use of black bean seeds by sickle cell patients. Mode of action, nutraceutical capsules/functional foods formulation with these phenolic compound extracts for SCD management will be the next step of this work.

#### Conflict of interest statement

We declare that we have no conflict of interest.

#### Acknowledgements

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