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Shilpa Vaishnava and V D Rangari
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Original Article

# *In-Vitro* Anti-Sickling Activity of Selected Medicinal Plant to Explore Herbal Remedies for Sickle Cell Anemia

Shilpa Vaishnava *, V D Rangari

SLT Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur Chhattisgarh-495001, India.

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ABSTRACT

**Objective:** To explore medicinal plants available in Chhattisgarh for its anti-sickling activity by using various in-vitro test and find a suitable herbal remedy for dreadful disease i.e sickle cell anemia.
**Methods:** Medicinal plants were extracted with suitable process and the dry extracts obtained were subjected to in-vitro antisickling activity by Emmel's test, Hemoglobin S solubility test/Hemoglobin polymerization, and osmatic fragility/Erythrocyte membrane stability activity
**Results:** The result of the activity indicated that the medicinal plants extracts *Wrightia tinctoria* and *Carica Papaya* have shown prominent activity while *Mangifera indica* and *Holarrhena antidysentrica* depited moderate activity for sickle cell disease.
**Conclusion:** Present Study conclude that for treating dreadful disease like sickle cell anemia herbal remedies can be one of the best approach, this study provides suitable herbal leads by providing its anti-sickling activity using various scientific models. Among various herbal extract results has indicated that the extract of *Wrightia tinctoria* (WTE) shows significant activity.
**Keywords.** Sickle cell, polymerization, deoxygenation, herbal plant.

## 1. INTRODUCTION

Each year, over 300,000 neonates born with sickle cell disease (SCD) and half of them die before the age of 5 years. This disease is more pronounced in developing countries [1] .The prevalence of sickle cell carriers found to be more among different tribal groups which varies from 1 to 40 % in India [2]. Majority of these tribal groups live in rural areas and not able to afford expensive proposed therapies such as the medullar implantation and repeated blood transfusion which increases our interest to investigate a novel and safe medicine for SCD. SCD is inherited chronic disease in

**Corresponding author ***
Shilpa Vaishnava
SLT Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur Chhattisgarh, India
E mail: shilpavaishnav3@gmail.com

which red blood cells (RBC) become crescent-shaped instead of disc-shaped. It is caused by mutation in the sequence of beta globin in 6th position, replacement of a polar amino acid (glutamic acid) by a less polar one (valine) leads to polymerization of hemoglobin S (sickle hemoglobin) in the red blood cells [3]. This aggregation modifies the shape of blood cells and makes them fragile and less flexible; that causes many complications in sicklers like hemolytic anemia, painful vaso-occlusive events, vascular remodeling, acute and chronic organ injury, and shortened lifespan [4].

Pathophysiological studies have shown that the dense, polymerized and dehydrated red cells may play a central role in acute and chronic clinical manifestations of SCD. During the deoxygenation which follows passage of RBCs in the microcirculation, the Hb molecule undergoes a conformational change [5]. In HbS, replacement of the hydrophilic group in the β-globin chain by the hydrophobic residue makes that this last one establishes hydrophobic interactions with other hydrophobic residues on the β-globin chain of another deoxy-HbS molecule [6]. Here due to lack of oxygen, deoxy HbS protein polymerize, leading to a rigid chain and induce the characteristic SS-RBC shape (sickle shape). This process needs a certain time to be primed, the so-called "delay time", which is inversely proportional to the intracellular concentration of HbS. One of the distinguishing characteristics of SCD is the presence of dense erythrocytes, formed as a result of cell dehydration and loss of potassium (K+) [7]. Normal RBCs cells keeps intercellular $Na^+$ and $Ca^{++}$ ions low and $K^+$ and $Mg^{++}$ ions high and the pathways are modulated by cellular energy. Due to ATP depletion, $Ca^{++}$ concentration increase 3-4 time in SCD patients [8]. Cells contain few or no endocytic vesicles for storage of large amount of $Ca^{++}$, it activate $Ca^{++}$-dependent $K^+$-channel (Gardos channel), loss of $K^+$ with accompanying movements of $Cl^-$ and water thereby resulting dehydration and hemochrome formation. It follows that loss of RBCs deformability and cell to cell adherence [9]. Several studies on pathophysiology of sickle cell indicate that anti-sickling agent may act on inhibition of polymerization or inhibit RBCs cell hemolysis.

Very few ethno medicinal remedies for the treatment of SCD have been reported in the literature due to secrecy attached to the treatments of this disease. Our present study was performed with the aim of screening the medicinal plants used to treat SCD in tribal area of Chhattisgarh state of India. The anti-sickling activity of the plants extracts were evaluated *in-vitro* on Sickle cell patient's blood using Emmel's test, polymerization inhibition assay and osmotic fragility test [10].

## 2. MATERIAL AND METHOD

**Survey and Collection of Plant Material**

The medicinal plant surveys conducted in the Achanakmar-Amarkantak region of Chhatisgarh state of India with the help of traditional healers known as Baigas and Vaida, and this survey provided us leads such as *Wrightia tinctoria* R.Br (Apocynaceae) (leaves), *Carica papaya* (Caricaceae) (unripe fruits), *Butea monosperma* (Lam.) kuntze (Fabaceae) (fresh flowers) , *Bombax ceiba* (Asclepiadaceae) (fresh flowers), *Mangifea indica* (Anacardiaceae) (leaves) and *Holarrhena antidysenterica* Wall (Apocynaceae), (seeds). These plants were collected from Chhatisgarh Medicinal Plant Board and the University Campus of Guru Ghasidas University Bilaspur. Plants were authenticated in Guru Ghasidas Central University, Bilaspur & voucher specimen were deposited at herbarium of Department of Botany (No. Bot/GGV/2018/25), in Guru Ghasidas Central University Bilaspur.

**Extraction**

*W. tinctoria* R.Br (leaf) (WTE), *M. indica* (leaf) (MIE) and *H. antidysenterica* (leaf) (HAE) were subjected to soxhlet extraction. 500 gm of the dried crude drug was taken and extracted for 72 hours using ethanol as a solvent. *C. papaya* (unripe fruit) (CPE),*B. monosperma* (fresh flower) (BME), *B. ceiba* (fresh flower) (BCE) were extracted by using maceration method. Fresh flower were collected and petals were separated from flower, crushed and placed in methanol for overnight in a refrigerator. After filtration, filtrates were concentrated and dried using rotary evaporator.

**Biological samples**

The blood samples used in the present study were provided by SRL Laboratories Bilaspur, theses sample were collected by the laboratory taken from adolescent patients known to have sickle cell disease in the Chhattisgarh area. None of the patients had been transfused recently with Hb AA blood. All anti-sickling experiments were carried out using a sodium citrate suspension of freshly collected blood. In order to confirm their SS nature, the above-mentioned blood samples were first characterized by hemoglobin electrophoresis on cellulose acetate gel at pH 8.5. They were confirmed to contain SS red blood cells and were stored at ± 4° C in a refrigerator.

**a) The Emmel's test**

Blood samples were kept in contact with plants extracts at different concentrations [with the physiologic solution (NaCl 0.9%, an isotonic solution of the cellular medium used to allow the osmotic regulation and hence to avoid precocious hemolysis of red blood cells) as the dilution solvent] according to Emmel's test procedure [11]. In this study, Emmel's test was performed as mentioned in Mpiana et al, 2007. A drop of physiological solution kept on a glass slide followed by addition of blood sample (one drop), glass slide were covered and kept for incubation for 24 hours in anaerobic conditions, which leads deoxygenation and transform them into sickle shaped. Glass slide were observed under microscope. The numbers of observed erythrocytes were determined using neubauer's cell.

**b) Hemoglobin S solubility test / hemoglobin polymerization**

HbSS polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm using 2% Sodium metabisulphite as reductant or deoxygenating agent [12]. 4.4 ml of 2% solution of sodium metabisulphite (Na2S2O3), 0.5 ml normal saline and 0.1ml of plant extract were pipetted into a cuvette, shaken and the absorbance was noted at 700 nm every five 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 extract and 0.1 ml of hemoglobin solution (HbSS) were pipetted into a cuvette and the optical density reading taken as above. The rates of hemoglobin polymerization were calculated from the formula of average change in optical density / absorbance against time in minutes [13].The rate of polymerization inhibition (% PI) versus time was calculated using the following formula:

$$\%\,PI = \frac{\text{Absorbance of untreated HbS - Absorbance of treated HbS}}{\text{Absorbance of untreated HbS}} \times 100$$

Before zero time (period of pre- incubation), the HbS which was soluble in aqueous medium was converted into deoxy-Hb form for which solubility is much reduced after chemical treatment by the sodium metabisulfite 2% (hypoxi) thus initiating a beginning of polymerization. At time zero, the absorbance of untreated HbS increases (due to the formation of polymer to tactoid which absorbs at 700 nm) where the absorbance of treated Hb decreases at the same wave length (inhibition of polymerization).

**c) Osmatic fragility / Erythrocyte membrane stability activity**

The osmotic fragility of erythrocytes measures the membrane stabilizing effect of the extracts in osmotic stress/hypotonic lysis incubation. To 10 mL reaction vessel containing 4 mL of different concentrations (0.00 - 0.85%) of buffered saline with pH of 7.4, 1 ml of each extract (250 µg/mL) and 0.05 ml SS-RBC blood were added. The mixture was incubated at room temperature (25°C) for 24 h and then centrifuged at 3000 rpm for 15 min. The optical density of the supernatant was read at 540 nm against blank made of 0.85% buffered saline concentration [(14)]. The mean corpuscular fragility (determined from the concentration of saline causing 50% hemolysis of the RBC) was obtained from a plot of lysis (%) *versus* NaCl concentration.

The effect of different herbal extracts on erythrocyte membranes was analyzed using the osmotic fragility test, which revealed appreciable membrane-stabilizing (protective) effects of the extracts and their inhibitory action on hemolysis of red blood cells. Red blood cells suspended in hypotonic salt (NaCl) solution take up water, swell, and become spheroidal and more fragile, and eventually burst. The increased fragility, which leads to lysis, is inversely proportional to the concentration of NaCl and directly proportional to the thickness of the red blood cells. An increase in osmotic fragility is equivalent to a decrease in osmotic resistance. The concentration of NaCl solution in which 50% lysis occurred is noted as the median corpuscular fragility (MCF).

## 3. RESULT

The results obtained from the above tests i.e. Emmel's test, Hemoglobin S solubility test and Erythrocyte membrane stability activity has been presented as follows.

**Effect of extract on sickle cell RBCs morphology (*Emmel's test*)**

Figure1.1 show the shape change in RBCs normal blood sample, negative controlled (untreated) and positive control (treated with *C. papaya* extract) and plant extract treated sample. Calculate average value of radius, perimeter and surface area of RBCs with different plant extracts is shown in table 1. This table indicates that where RBCs with the plant extract shows change of sickled shape to round shape. This statement confirmed by changes in radius shape and both surface area and perimeter of treated cells [15]. The results confirm that tasted plants have antisickling activity ($p < 0.05$). The results are presented in Table 1 and Fig. 1-8.

**Table 1: Average values of radius, perimeter and surface of erythrocytes before and after treatment with plant extracts**

| S. no. | Sample and code name | Surface (µη2) | Perimeter (µη) | Radius (µη) |
|---|---|---|---|---|
| **1** | Negative control (NC) | 19.2±1.0 | 31.7±1.5 | -------- |
| **2** | *C. papya* extract (CPE) | 31.8±3.22 | 18.4±2.3 | 3.1±0.55 |
| **3** | *W. tinctoria* extract (WTE) | 33.72 ± 1.7 | 19.39 ± 1.1 | 3.3 ± 0.3 |
| **4** | *H. antidysenterica* extract (HAE) | 29.2 ± 3.26 | 20.23 ± 2.19 | 3.17 ± 0.43 |
| 5 | *M. indica* extract (MIE) | 21.1± 2.14 | 32.9± 3.14 | ------- |
| 6 | *B. monosperma* extract(BME) | 20.6± 1.23 | 33.0± 1.3 | --------- |
| 7 | *B. ceiba* extract (BCE) | 20.2± 6.2 | 31.9± 1.42 | -------- |

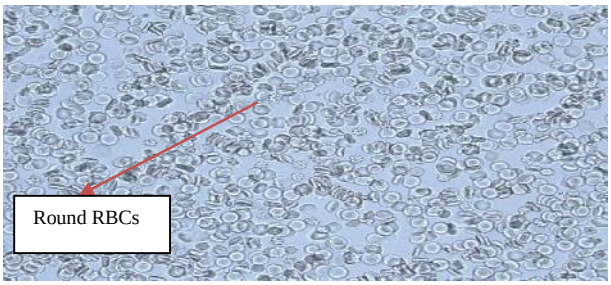


**Fig 1: Normal blood cells**

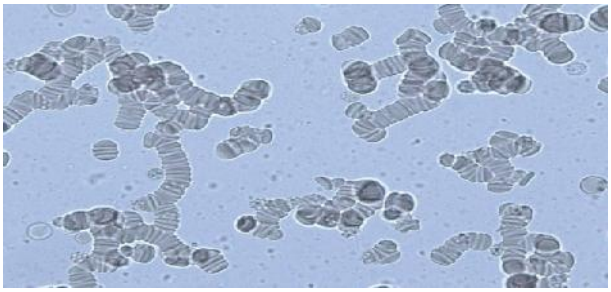

**Fig 2: Negative control sickled cells**

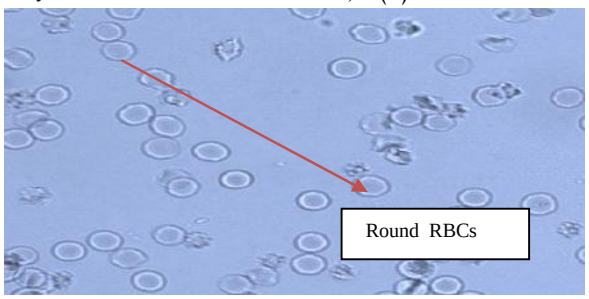


**Fig 3: Treated with CPE (positive control)**

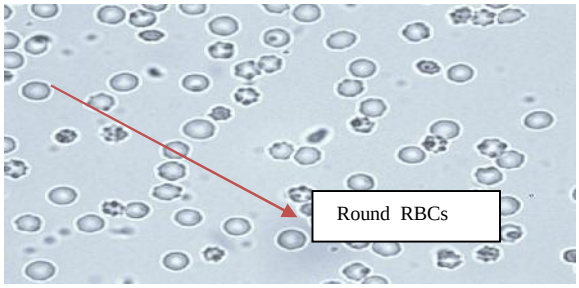


**Fig 4: Treated with WTE**

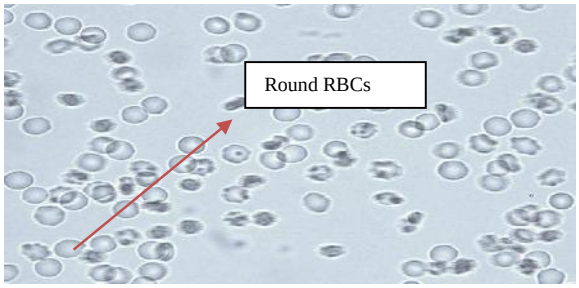


**Fig 5: Treated with HAE**

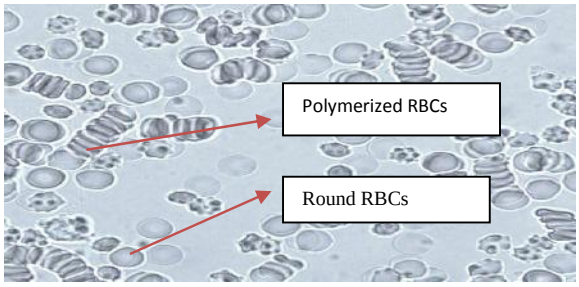


**Fig 6: Treated with MIE**

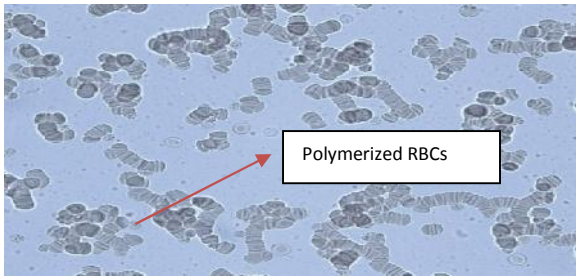


**Fig 7: Treated with BME**

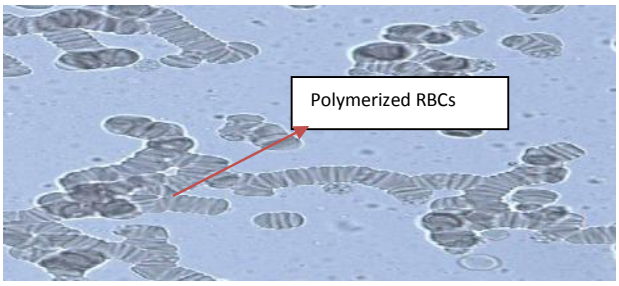


**Fig 8: Treated with BCE**

*Fig 1-8 Result for Emmel's test (Blood sample treated with different plant extract)*

**Effect of extract on Polymerization of RBCs**

The effect of extract on the polymerization of deoxy-HbS can be determined by studying the solubility of deoxy-HbS in the absence and in the presence at 250mg/ml concentration of plant extracts. This was done by monitoring the polymerized HbS at 700 nm at different time interval. Graph shows the changes in polymerization with time interval. Negative control, positive control and different plant extract shown in graph 1.

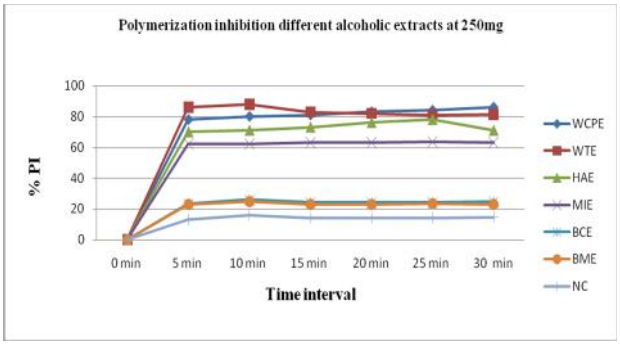


**Fig 9: Polymerization inhibition shown by extracts**

**Effect of extracts on osmatic fragility test:**

The effect of different plant extracts on the membrane stability of RBC can be evaluated by comparing the percentage of hemolysis of untreated and treated SS RBCs using the osmotic fragility test. Graph 2 shows the percentage lysis of untreated and treated SS RBC at different saline concentrations.

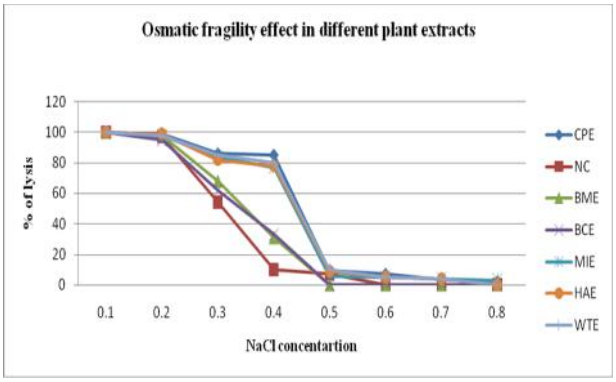


**Fig 10: Osmotic fragiliogram after treatment with different plant extracts.**

## 4. DISCUSSION

Fig. 1 shows normal condition of RBCs untreated sodium metabisulfite hypoxia condition. All RBCs are disc shaped and healthy in appearance. Fig. 2 is from negative control treated sodium metabisulfite but not treated with any drug. Fig. 3 positive control treated with sodium Meta bisulfite then *C. papya* extract used as a standard here and can see significant changes in RBCs as compare to negative control. Fig. 4 (WTE) and 5 (HAE) shows the majority of the erythrocytes recovered in normal shape like positive control. Fig 6 (MIE) shows less recovery of RBCs and fig 7 (BME) and 8 (BCE) are not that much recovery. So we cannot consider sample BME & BCE effective in sickle cell. As it can be seen, a normalization of sickle cell treated with WTE, HAE & MIE. It was reported that unripe *Carica papaya* extract used for normalizing the sickle cell erythrocytes [16]. Here the WTE result is almost similar to CPE. From the above result it can be assured that the activity of *W. tinctoria* extract (WTE) is almost similar with *C. papaya* extract.

Above results are statically treated according to Tukey's multiple range test [17]. That enables the determination of a significant difference between the untreated and treated group. Shape normalization can be quantitatively evaluated from the values of parameters such as (1) the surface area (in mathematical analysis surface area of round cells is higher than sickle shape and study literature [18] says that higher surface area increase the capability of carrying oxygen), (2) the perimeters ( when round shape change in sickle shape it shrink from middle and elongated from corner thus the value of perimeter is higher in sickle than round) and (3) the radius ( radius only possible to count when cell is round shape not apply for sickle shape) . Based on above theory it can be observed from the results given in table-1 that the perimeters of untreated RBCs are higher than those of the treated groups with WTE and HAE. Surface area and radius of cells are higher in treated group compare to untreated group. Because of the sickle shape not change in the samples treated with MIE, BCE, BME and untreated RBCs, software not give those groups RBCs radius result. Based on all three parameter result shows that antisickling activity of WTE is higher than to CPE (positive control). However HAE show positive activity near to CPE. Remaining extract does not show effect on sickle cells. The above results are in agreement with our findings on shape of RBCs that revalued that biconcave shape higher the surface area higher oxygen caring capacity [19]. These result agreement for bioactive plants and confirm the antisickling activity of WTE and HAE.

Pathophysiology of SCD has been suggested to change in sickle hemoglobin polymerization. Literature [20] says polymerization of deoxy HbS reduce its oxygen affinity and cause sickling. We can be predict that higher the percent of polymerization inhibition (PI), higher is oxygen affinity. Higher the oxygen affinity we compare the result of different extract it shows maximum difference between negative control and positive control groups. WTE extract has shown better activity than standard positive control. HAE & MIE showed moderate % PI activity as compared to earlier one. Someplace BCE and BME showed less than 50% of PI. In this paper we discus about the delay time. This delay time is sensitive to Hb polymerization. In SCD therapy longer delay time,decrease the polymerization and decrease the probability of RBCs sickling [21]. In graph we can see delay time is between 0 to 5 min. that maximum changes need in polymerization is between this times then the graph will continue with small changes. Longer delay time reduce the polymerization [22]. Therefore it can be assumed that, anti-sickling activity seems to be achieved by the direct interfering in delay time.

Sickle erythrocytes have been reported to have a distorted volume-to-surface ratio when compared to normal erythrocytes and so a shift to the left in the osmotic fragiliogram suggests a higher osmotic resistance for most sickle cells. This shift was observed in the study, showing that the extracts were able to protect the integrity of the erythrocyte membrane by, increasing its resistance to osmotic stress/lysis, and thus reducing membrane fragility. From these erythrocyte studies, it can be reported that aqueous extract of *C. papaya* reduced hemolysis and confirm some protective effect on erythrocyte membrane. This shift was observed in the study, showing that the extract was able to protect the integrity of the erythrocyte membrane, increase its resistance to osmotic stress/lysis, and thus reduce membrane fragility. In graph 2 indicate higher percent of lysis is higher in CPE, WTE and HAE as compared to negative control. That indicates that higher the osmotic fragility means higher the rounded cells of RBCs [23].

## 5. CONCLUSION

Results of this study has indicated that the ethanol extract of *Wrightia tinctoria* (WTE) shows significant or better antisickling activity as compare to the standard *Carica papaya* extract (CPE). MIE and HAE showed moderate activity. BCE and BME failed to show antisickling activity in these *in- vitro* studies. The ability of the extracts, in this study, to normalize the SS blood erythrocytes, polymerization inhibition and osmotic fragility may represent a rational explanation for the use of these plants *W. tinctoria* seeds or *C. papaya* in treating SCD. These species have not yet been reported to exhibit antisickling effects. Further studies are, therefore, necessary to evaluate the potential of these plants as effective antisickling agents.

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