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# Antisickle Erythrocytes Haemolysis Property, Polymerization Inhibition and Phytochemical Analysis of the Endophytic Extract of *Justicia secunda* Leaves

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## Abstract

*Justicia secunda* is used locally for wound healing, anemia, abdominal pain, etc. Endophytes are reported to produce bioactive metabolites. The study aimed to perform pharmacognostic analysis and evaluate the effect of endophytic extract of *J. secunda* on human sickle haemoglobin and polymerization inhibition. Plant samples were collected and dried under shade, pulverized. Chemomicroscopy was conducted. After surface sterilization, samples were air dried, cut into sizes and aseptically cultivated on sterile malt extract agar plates, incubated at 25-28 ºC for 4-7 days. Pure endophytic fungi were fermented on rice medium. With ethical approval, blood samples were collected from the sickle cell individuals. HbSS polymerization was assessed by the turbidity of the mixture at 700 nm. There was significant percentage antisickling of 63.22% at 5 mg/mL, 53.90% at 2.5 mg/mL and 50.44% at 1.25 mg/mL of the endophytic extracts. The endophytic extract (LB2) reduced sickled cells compared to the control. The polymerization was reduced from 1.880-1.839 by the 5% w/v extract, 1.587 to 1.534 by 2.5%w/v extract etc., in more than 30 min. The endophyte extract of *J. secunda* may be used in managing anaemia in patients with sickle cell disease.

***Keywords:*** *Justicia secunda, Anemia, Sickle cell, Medicinal plant, Red blood cells*



## 1. Introduction

Sickle Cell Disease (SCD) is a genetic blood disorder characterized by abnormal rigid sickle shaped Red Blood Cells (RBCs). Sickling decreases the RBC's flexibility and results in a risk of various complications. It is the most prevalent

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human hereditary disorder with prominent morbidity and mortality. It is due to the change of an amino acid in position six within the beta globin chain of hemoglobin molecule whereby glutamic acid is replaced by valine (Serjent and Serjeant, 2001). At low oxygen tension, the mutant hemoglobin polymerizes inside the RBCs into a gel or further into fibers leading to a drastic decrease in the red cell deformability. Polymerization and precipitation of sickle hemoglobin (HbS) within the erythrocytes cause the change of shape from the normal spherical form into the one resembling a sickle. A single nucleotide substitution (Thymine [T] for Adenine [A]) allows HbS to polymerize when deoxygenated, since valine can dock with the complimentary sites on the adjacent globin chains (Eaton and Hofrichter, 1987). Some live a normal life while others suffer from a variety of life-threatening complications. Typical symptoms of SCD are anemia, episodes of pain, swelling of hands and feet, frequent infections, delayed growth or puberty, vision problems (WHO, 2005).

Plants are natural sources of a vast number of bioactive compounds known for their therapeutic and physiological properties (Mustafa and Wardah, 2014). In South-Eastern Nigeria, Congo and South Côte-d'Ivoire the leaf decoction of *Justicia secunda* is used in the management of anemia (Koffi *et al*., 2012). Earlier studies on haematinic, antimicrobial and anti-hypertensive activities of *J. secunda* have been reported (Fongod *et al*., 2013; Anyasor *et al*., 2019). Phytochemical screening of the plant has shown the presence of tannins, flavonoids, alkaloids, quinines and anthocyanins (Lindo *et al*., 2012). Luteolin, aurantamide acetate, auranamide, quindoline and secundarellone have been isolated from *J. Secunda* (Koenig *et al*., 2012; Onoja *et al*., 2017). Notwithstanding the popular uses of *J. secunda* in folkloric medicine only few pharmacological studies have been done on the plant (Koenig *et al*., 2012). Compared to many plants whose iron contents are known, *Justicia secunda* appears to be a very important source of iron. These high iron contents (240 mg / g $\pm$19) justify its use as anti-anemic agent in the Congolese popular medicine (Moswa *et al*., 2008).


**Figure 1: Leaves and Flower of *Justicia secunda***

The leaf of the plant is purposely consumed with its characteristic red color decoction for packed cell volume improvement in certain parts of Nigeria, Congo and Cote-d'Ivoire (Mpiana *et al*., 2010). Extracts made from only the leaves are the most used, followed by those extracts made from only the roots. The leaves are prepared as a decoction for the management of anaemia and as an antioxidant in stress management. Earlier studies confirmed the haematinic, anti-sickling, anti-hypertensive and antimicrobial potentials of the plant (Koffi *et al*., 2012). Zambrano *et al.* (2017) showed that quindoline, a constituent of *J. secunda* stem, has only very minimal acetylcholinesterase inhibitory activity *in vitro* and so can help in muscle activation and contraction (Khan *et al*., 2019).

Endophytic fungi are microorganisms found in almost all plant parts and act as chemical synthesizers in the host plant, producing a wide range of bioactive secondary metabolites (Ibrahim *et al*., 2021). Endophytic fungi have been previously identified and their potential explored to produce bioactive compounds with useful pharmaceutical applications (Nwankwo *et al.,* 2021). Endophytic fungi from the genera *Colletotrichum, Fusarium, Alternaria* and *Aspergillus,* isolated from medicinally important plants exhibit a variety of biological activities such as anticancer, antimicrobial, antifungal, immunomodulatory, antitubercular, and antioxidant activities with wide application in agrochemical and pharmaceutical industries (Garima and Mukesh, 2021). These biological activities demonstrated by endophytes have been attributed to isolated and identified secondary metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, peptides, and phenols present in the fungal extracts (Strobel, 2002). The main factors that may regulate entophyte colonization within a plant or microbial species, include the genotype of the plant, the growth stage of the plant, the physiological status of the plant, the type of plant tissues, the environmental condition of the soil in which it is grown, the sampling season, the surface sterility, selective media and culture conditions as well as different agricultural practices (Gaiero *et al*., 2013). Extraction of metabolites from endophytes is affected by factors, such as the season of sample collection, climatic condition and geographical location (Shukla *et al*., 2014). Many endophytic fungi have been proven to have the ability to produce novel secondary metabolites to overcome pathogenic invasion. Interestingly, the host-endophytic relationship is found to be complex and varies in different hosts or microorganisms (Tan and Zou, 2001; Pullen *et al*., 2002). Various extracts of *Justicia secunda* including anthocyanin and aqueous extracts have been reported to show antisickling, antimicrobial and anti-hypertensive activities (Ngbolua *et al*., 2010). However, only very few studies have been performed on the endophyte extract of *J. secunda*. This study was aimed to carry out the pharmacognostic standardization and evaluate the effect of the endophytic extract of *Justicia secunda* (Acanthaceae) on human sickle haemoglobin and polymerization inhibition. Thus we tried to solve the problem of plant preservation through the use of plant endophyte extract instead of crude plant extract which involves a large amount of plant itself.

## 2. Method

### *2.1. Fresh Leaf Microscopy*

Samples of the plant under study were collected from Agulu, Anambra state, Nigeria. The leaves of *Justicia secunda* with no disease condition were harvested from Agulu, Anambra state, Nigeria and identified by a plant taxonomist Ms. Ike Obiageli in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka. The voucher specimen PCG474 / A /078 was deposited at the herbarium of the department of pharmacognosy and traditional medicine. Foliar epidermis of the adaxial (upper surface) and abaxial (lower surface) surfaces of the leaves were prepared by clearing method. The leaf samples were cleared by soaking in commercial bleach "Hypo" containing 3.5% sodium hypochlorite for 18 h. Then, the epidermal strips of the leaf samples were scrapped gently with the aid of a pair of forceps and placed on a clean slide, and then stained with Safranin solution and covered with a cover slip (Nwafor *et al.,* 2019). The slides were viewed under a light phase contrast microscope (Motic B3, Motic Carlsbad, CA, USA) at x 40, x 100 and x 400 magnifications and photomicrographs were taken with a Moticam 2.0 image system with software (Motic Carlsbad, CA, USA) fitted to the microscope. The following parameters were observed and assessed:

- Epidermal Cells: The type and number of epidermal cells were counted and recorded.
- Stomata Type: The stomatal complex types were observed and recorded following the terminologies of Evert (2006).
- Stomata Size (length and width): The stomata length and width were measured using Motic microscope software a total of 10 fields of views for each sample.
- Stomatal Density: The stomatal density was determined as the number of stomata per square millimeter.
- Stomatal Index: The stomatal index was determined as follows:

$$SI = \frac{S \times 100}{S + E}$$

where, S = Number of stomata in a field of view

E = Number of epidermal cells in the same field of view

- Trichome Parameters: The trichome types, size, density and index were determined following the same procedures as the stomata above.
- Vein islet number, vein islet termination number and palisade ratio.
- All parameters were observed on both the adaxial and abaxial surfaces of the leaves (Nwafor *et al.*, 2019).

Transverse Section (TS) of the leaf was made using a Reichert sledge microtome following the procedures of Johansen (1950) and Nwosu (2006). The sections were microtomed at 10-15 unimicrons and were picked with the aid of a camel hair brush from the tip of the microtome knife into separate Petri dishes containing 70% absolute alcohol and labeled appropriately. Safranine and Fast green served as biological stains in differentiating lignified tissues.

## 3. Chemomicroscopy

The leaves were dried under shade and pulverized with local mortar and pestle. Chemomicroscopy conducted on the powders to determine the presence of starch, calcium oxalate crystals and lignified vessels. A judicious quantity of the sample was dropped on a glass slide. One drop of chloral hydrate was dropped and passed over a Bunsen burner repeatedly until bubbles formed. This signified the successful clearing of the tissues.

### *3.1. Isolation, Purification and Fermentation of Endophytic Fungi*

The harvested samples were washed thoroughly under running tap water and air dried before culturing under aseptic conditions. Several endophytic fungi were isolated according to the protocol previously reported by Okezie *et al*. (2020), which was modified slightly. The samples were subjected to a four-step method of surface sterilization process in other to rid of contaminants on the surface of the samples which includes washing using distilled water then subjected to surface sterilization by immersion for 2 min in 2% v/v hypochlorite solution and 1 min in 70% v/v ethanol, and further washed three times in sterilized double distilled water for 3 min. After surface sterilization, the samples were air dried then cut into sizes and aseptically cultivated on sterile Malt Extract Agar (MEA) plates supplemented with 50 µg/mL chloramphenicol to suppress bacterial growth. The cut end of the material was made to contact the medium. The plates were incubated at 25-28 ºCfor 4 to 7 days on the bench with normal day lights and dark periods. Hypha emerging out of the cultured material were sub-cultured multiple times in order to get axenic cultures. Pure endophytic fungi were fermented on rice medium for 21 days.

### *3.2. Extraction of Secondary Metabolites*

At the completion of the fermentation, 500 mL of ethylacetate were transferred into each fermentation flask, homogenized and agitated for two days. Following the stoppage of fermentation process, the homogenate were filtered and concentrated at a reduced temperature of 50 ºC using a rotary evaporator. Each fungal extract was kept in the desiccator for proper drying and the weights recorded. The dried extracts were thereafter kept in the refrigerator at 4 ºC prior to further analysis.

### *3.3. Blood Sample Collection*

An ethical approval was obtained prior to commencing this study from Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State. Fresh blood samples were collected with full informed consent from the sickle cell individual in the steady state of the disease, aged 25 years, who had not taken any herbal medication for SCD within the past six months.

### *3.4. Preparation of Sickle Cell Blood*

Venous blood samples (5 mL) were collected in sodium EDTA bottles. Erythrocytes were isolated from whole blood by centrifuging at 1500 x g for 15 min. The erythrocyte sedimented while the plasma was siphoned out carefully using a Pasteur pipette. By repeated inversion, the erythrocytes were suspended in a volume of isotonic saline equivalent to the siphoned plasma. The suspended erythrocytes were used for the antisickling test while some was freeze thawed in a freezer to release a hemolysate. The hemolysate was used for the haemoglobin polymerization experiment (Nwaoguikpe and Braide, 2012).

### *3.5. In vitro Anti-sickling Activity of the Extracts*

A serial dilution of different concentrations of aqueous extracts of *Justicia secunda* was prepared in the saline solution. For the assay, 100 μL of SS-RBC was pre-incubated with 100 μL of 2% sodium metabisulphate and 100 μl each of a solution of the different concentrations of the extracts for different concentrations of 125, 250, and 500 μg/mL. Each mixture was incubated at 37°C for 2 h (time necessary to obtain maximum sickling). After incubation, 10 μL of the mixture was diluted 100 fold. A drop of each sample was examined microscopically (Amscope, USA) using a magnification of X100. 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 phosphate saline solution.

The percentage of sickling was calculated using the formula (Nwaoguikpe and Braide, 2012).

$$\% \text{ of sickling} = \frac{\text{Number of sickled cells}}{\text{Total cells}} \times 100$$

### *3.6. Sickle Cell Hemoglobin Polymerization Test*

The original methods of Noguchi and Schechter (1989) as reported by Nwaoguikpe and Braide (2012) was used for the HbSS polymerization experiment. HbSS polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm using 2% solution of Sodium metabisulphite as a reductant or deoxygenating agent. A 4.4 mL volume of 2% Sodium metabisulphite, 0.5 mL of normal saline (0.9% NaCl) and 0.1 mL of haemoglobin were pipette into a cuvette, shaken and absorbance read in a Spectrophotometer at 700 nm for 30 min at 5 min intervals. This served as control. For the test assay, 0.5 mL normal saline was replaced with 0.5 mL antisickling agent (extract of *Justicia secunda*) and readings taken as usual. The rates of polymerization was calculated from the formula of average change in absorbance against time in minutes.

For the polymerization test:

$$Rp = \frac{pf - pi}{t}$$

where, *Rp* = rate of polymerization, *pf* = final absorbance, *pi* = initial absorbance at time zero, *t* = time of reaction in minutes.

## 4. Results

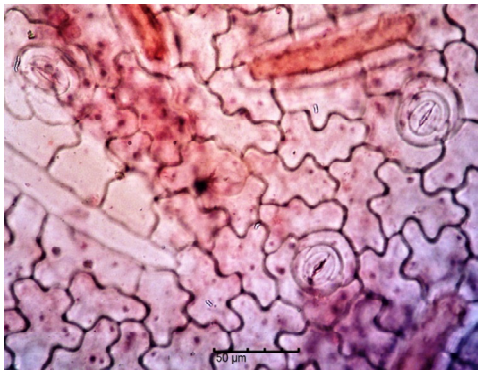
### *4.1. Microscopy*

**Table 1: Summary of the Fresh Leaf Microscopy of *J. secunda***

| | |
|---|---|
| Epidermal cell | The epidermal cells are irregular in shape with undulated/wavy anticlinal cell wall on both the upper and lower surfaces. |
| Stomata type | The leaf is amphistomatic (stomata occur both on the upper and lower surfaces but more on the lower surface) with diacytic type of stomata (two subsidiary cells lie adjacent to the guard cells) |
| Trichome | Covering multicellular trichomes are scarcely present. |
| Stomata density ($mm^{-2}$) | Upper surface: 19.12 ± 1.47; Lower surface: 70.00 ± 1.47 |
| Stomata length (μm) | Upper surface: 22.33 ± 1.96; Lower surface: 25.47 ± 0.62 |
| Stomata width (μm) | Upper surface: 16.51 ± 0.17; Lower surface: 13.87 ± 0.41 |
| Stomata index (%) | Upper surface: 7.35 ± 0.58; Lower surface: 29.15 ± 0.64 |
| Stomata size ($\mu m^2$) | Upper surface: 369.35 ± 35.50; Lower surface: 353.51 ± 15.21 |
| Palisade ratio | 13.54 ± 0.25 |

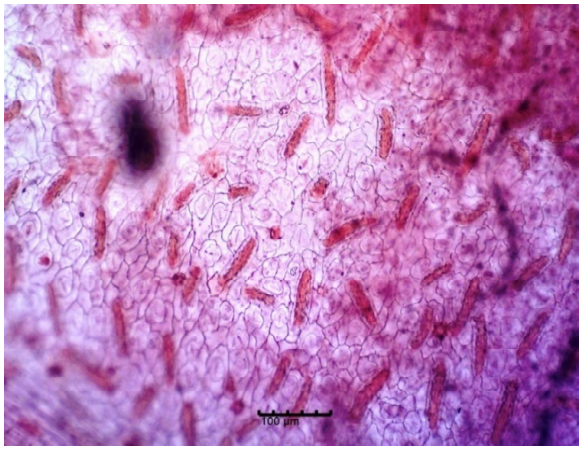
**Table 2: Result of Powder Microscopy of *J. secunda* Leaf**

| Parameter | Reagent(s) | Result |
|---|---|---|
| Starch grains | Iodine solutuion | Present |
| Lignified tissues | Conc. HCl + Phloroglucinol | Present |
| Calcium oxalates | Iodine solution<br>Conc. Sulphuric acid | Present; Prism shape |
| Tannin | Ferric chloride | Present |
| Cellulose | Zinc chloride;<br>Conc. Sulphuric acid | Present |
| Gum / Mucilage | Ruthenium red | Absent |
| Protein | Biuret reagent;<br>Nihydrin | Present |
| Oil globules | Sudan IV reagent | Absent |



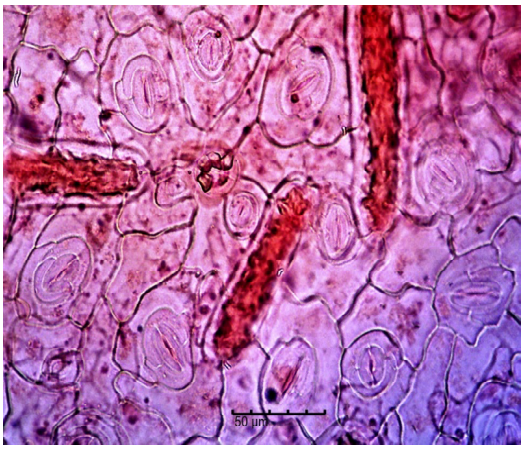

X100

**Plate 1: Upper Epidermal Surface of the Leaf of *J. secunda* Showing Undulated Epidermal Cells and Diacytic type of Stomata**



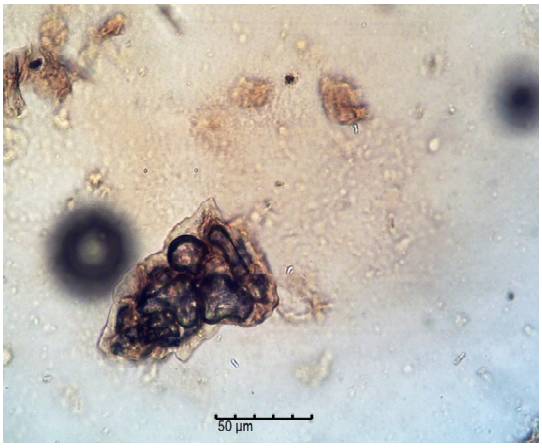

X400

**Plate 2: Lower Epidermal Surface of the Leaf of *J. secunda* Showing Abundant Secretory Tissues**




X400

**Plate 3: Lower Epidermal Eurface of the Leaf of *J. secunda* showing Undulated Epidermal Cells and Diacytic Type of Stomata. Secretory Tissues are also Prominent**

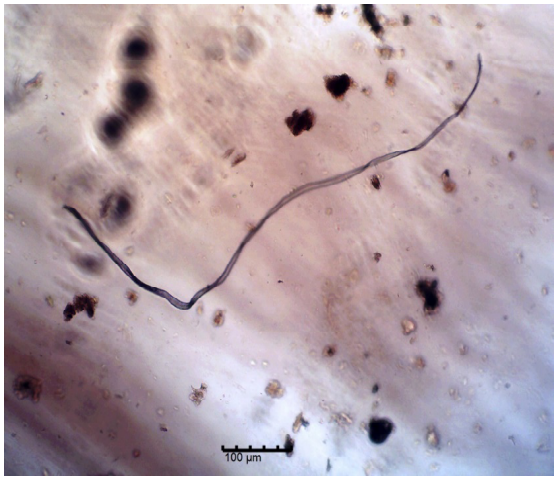



X400

**Plate 4: Chemomicrograph of the Leaf Powder Showing Cluster of Calcium Oxalate Crystals**


X400

**Plate 5: Chemomicrograph of the Leaf Powder Showing Multicellular Covering Trichome**




X400

**Plate 6: Chemomicrograph of the Leaf Powder Showing an Isolated Strand of Fiber Element**

### *4.2. Phytochemical Analysis*

The phytochemical analysis of the leaf of *Justicia secunda* showed the presence of Phenolics, flavonoids, Tannins, Alkaloids, Saponins and Terpenoids but cyanogenic glycoside was absence.

## *5.* Discussion

The medicinal potential of any plant is measured by its ability to reduce or mitigate the harmful effects of a disease. The therapeutic effect of such plant is also related to the various bioactive components that are naturally in the plant and their endophytes. The microscopic examination of the leaves of *Justicia secunda* showed the presence of an irregular shaped epidermal cell with undulated / wavy anticlinal cell wall on both the upper and lower surfaces (Table 1). Presence of stomata which is amphistomatic (stomata occuring both on the upper and lower surfaces but more on the lower surface) with diacytic type of stomata (two subsidiary cells lie adjacent to the guard cells). Covering multicellular trichomes are scarcely present. The chemomicroscopic examination of the powdered leaves of *Justicia secunda* showed the presence of starch, lignified tissues, calcium oxalate crystals, Cellulose and protein, fibre. The phytochemical analysis of the leaves showed the presence of phenolics, flavonoids, tannins, alkaloids, saponins and terpenoids (Table 2 and Plate 1 to 6). The presence of flavonoids, tannins, glycosides and saponins in the present study agrees with earlier reports (Adams *et al*., 2007), while the six phytochemicals present compared well with other reports on *J. secunda* leaves as well as *J. carnea* leaves (Table 3) (Onyeabo *et al*,. 2017). This research has

**Table 3: Result of the Phytochemical Screening**

| Phytochemical | Qualitative Screening | Quantitative Determination (mg/g) |
|---|---|---|
| Phenolics | + | 22.47 ± 1.24 |
| Flavonoids | + | 6.44 ± 0.44 |
| Tannins | + | 16.78 ± 1.66 |
| Alkaloids | + | 12.04 ± 0.88 |
| Saponins | + | 7.32 ± 0.32 |
| Terpenoids | + | 9.36 ± 0.92 |
| Cyanogenic glycoside | - | - |

**Note:** + present; - absent. Values are expressed as mean ± standard error.

confirmed that herbal drugs can be very useful in management of certain diseases as reported by Ogechukwu *et al.* (2022).

### *5.1. Sickling Reversal Effect*

Sickle cell disease affects the shape and flexibility of RBCs in such a way that it prevents their smooth movement through small human blood vessels. Normal red blood cells are biconcave and flexible, a property that enables them to move freely and smoothly through narrow blood vessels. It also enables them to live longer to about 120 days. One of the motives for antisickling drug design is to have a drug that can prevent or reverse the sickle shape phenotype of the RBCs (Nurain *et al*., 2017). There was a significant percentage antisickling of 63.22% at 5 mg/mL, 53.90% at 2.5 mg/mL and 50.44% at 1.25 mg/mL of the endophytic extracts (Table 4 and Figure 2).

**Table 4: Table Showing the No. of Total and Sickled Red Blood Cells**

| Sample | Total No. of Cells | No. of Sickled Cells |
|---|---|---|
| 500 mcg/mL | 154.00+28.50 | 12.00+6.00 |
| 250 mcg/mL | 138.50+17.50 | 14.50+1.50 |
| 125 mcg/mL | 154.00+7.00 | 15.00+2.00 |
| Control | 132.50+9.50 | 28.00+1.00 |

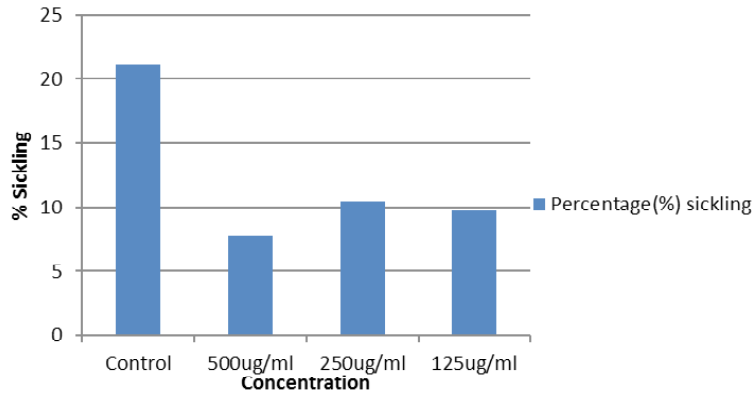



**Figure 2: Inhibition of Sickling of RBCs by Endophyte Extract of *J. secunda***

The endophytic extract of *J. secunda* (LB2) showed a reduction of the sickled cells with respect to the control from 21.13% to 7.77% by the 5%w / v *J. secunda*, from 21.13% to 10.47% by 2.5%w / v extract and from 21.13% to 9.74% by 1.25%w / v extract. This indicates that *Justicia secunda* has the ability to reverse sickling of red blood cells. Figure 3 shows the structural shape of sickled (a) normal; and (b) red blood cells when viewed under microscope at X 100 magnification. When the SS red blood cells were mixed with an endophyte extract of the plant, the majority of the erythrocytes recovered a normal shape. These morphological changes were observed in hypoxic conditions, i.e., after deoxygenation of haemoglobin. This morphological normalization of SS erythrocytes following treatment with endophytes extracted from *Justicia secunda* indicates the influence of the extract on the propensity to sickling of the RBC (Plate 1 to 6).

### *5.2. Red Blood Cell Polymerization Inhibition*

It has been well established from research in the past that the genetic mutation in the globin chain is where the sickle cell disease originated. One of the clinical manifestations of this genetic RBC disorder is polymerization

of haemoglobin in the hypoxic condition (Nagel *et.al.*, 1979). Therefore, inhibition or prevention of haemoglobin polymerization is one of the avenues of drug design against sickle cell disease. Figure 4 presented the effect of endophyte extract of *Justicia secunda* on sickle cell RBC polymerization inhibition. The initial absorbance of the polymerizing cells was measured at time zero (i.e., immediately after addition of sodium metabisulfite) and subtracted from the final absorbance taken at 30 min. The resulting value divided by 30 gives the rate of polymerization inhibition.

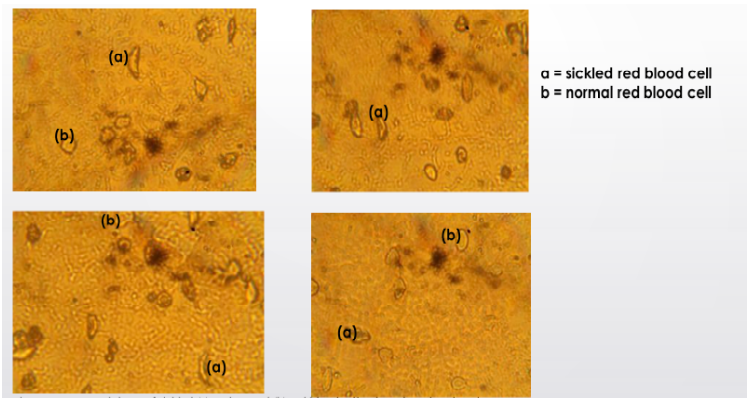



**Figure 3: Structural Shape of Sickled (a) Normal; and (b) Red blood cells When Viewed Under Microscope at X 100 Magnification**

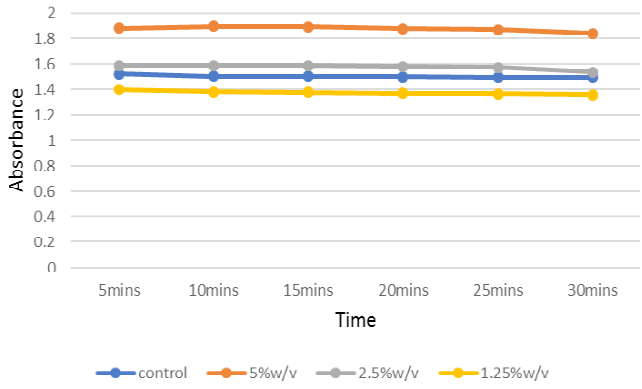



**Figure 4: RBC Polymerization Inhibition Rates by Endophyte Extracts of *J. secunda***

From Table 5 it can be deduced that the polymerization rate in the presence of 2% sodium metabisulphite and the endophyte extract of *J. secunda* was reduced with time from 1.880 to 1.839 by the 5% w / v extract, from 1.587 to 1.534 by 2.5%w / v extract and from 1.397 to 1.358 by 0.0125%w / v over 30 min. It is observed in Figure 4, that 5%w / v endophyte extract of *J. secunda* possesses the fastest rate in hemoglobin polymerization inhibition followed by the 2.5%w / v of the extract. It can be inferred from the graph in Figure 2 that some of the bioactive components in the endophyte extract of *J. secunda* was able to interact with hemoglobin molecules (two or more amino acid residues) or the RBCs membrane to bring about inhibition of the polymerization.

**Table 5: Red Blood Cell Polymerization Inhibition Test: Absorbance of Different Concentrations of Extract (JS-LB2) with respect to Time**

| Time (min) | Control | 5%w/v | 2.5%w/v | 1.25%w/v |
|---|---|---|---|---|
| 5 | 1.522 | 1.880 | 1.587 | 1.397 |
| 10 | 1.501 | 1.893 | 1.588 | 1.379 |
| 15 | 1.501 | 1.890 | 1.587 | 1.376 |
| 20 | 1.498 | 1.875 | 1.580 | 1.368 |
| 25 | 1.493 | 1.867 | 1.573 | 1.364 |
| 30 | 1.493 | 1.839 | 1.534 | 1.358 |

## 6. Conclusion

The endophyte extract of *Justicia secunda* was able to reduce the percentage of sickle cell and slightly reduce / inhibit the rate of hemoglobin polymerization of human sickled RBCs. The ability of this endophyte plant extract to exhibit these properties is probably due to the presence of the identified phytochemicals (bioactive agents). Thus, endophyte extract of *J. secunda* may be used in management of anaemia in patients with sickle cell disease.

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