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Phytochemical Analysis and Biomedical Potentials of White Stickpea (*Zapoteca portoricensis* (Jacq.) H.M. Hern Stem Bark

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ABSTRACT

White Stickpea (Zapotecaportoricensis (Jacq.) H.M. Hernbelongs to the family of Fabaceae/Miniosideae. It used as a traditionalremedy against infections that cause tonsillitis, fever, and breast enlargement. Some botanical compounds of this plant reportedly have medicinal importance. However, whether this plant can induce anti-sickling effects is yet to be assessed. The genetic disease Sickle cell anemiais common in African populations famous for its selection for protection in heterozygotes against malaria, but in homozygotes lead to serious complications. In this study we were interested in investigating thechemical properties of the traditionally used medicinal plant, Zapotecaportoricensis. Using microscopy and gas chromatography flame ionization detection (GC-FID) respectively, we report an ability of Stickpea extracts to revert sodium metabisulfite-induced sickle red blood cells to assume the shape of normal red blood cells. The reversal was 85.3% of the cells when 250ug/ml of the ethanol extract of Zapotecaportoricensis stem bark was present for 10 minutes, compared to the control which retained 90.74% sickle cells. Different concentrations of plant extracts, i.e500µg/mland 1000µg/ml of the plant extracts were assessed and significant results obtained. Indeed analysis of these extracts revealed the presence of bulky aromatic compounds, such as butein, genistein, epicatechin, ellagic and vanillic acid, suggesting that its anti-sickling mechanism might involve the interaction of these hydrophobic compounds with the deformed plasma membrane of sickle cell or interact with the valine or other hydrophobic amino-acid residues on the β-subunit of hemoglobinwhich can affect the structure of sickle red blood cells.

Keywords: *Medicinal, Phytochemical, Anti-sickling, White Stickpea, Zapoteca,Hemoglobin.*

INTRODUCTION

White Stickpea *(Z.*portoricensis) is a species from the *Fabaceae* family, a smooth deciduous shrub with thin, unarmed branches. In eastern Nigeria, locals commonly refer to it as Elugelu and use its roots and leaves to treat tonsillitis, spasms, and other gastrointestinal problems [32]. *Z. portoricensis*, commonly known as the white stickpea, has traditionally been used in medicine to alleviate conditions including constipation, convulsions, madness, skin infections, and external wounds [2]. This plant is known for its anti-inflammatory, antimicrobial, anti-ulcer, and antioxidant properties [1,2,3,23]. Plants naturally produce chemical compounds known as primary and secondary metabolites during their metabolic activities [26]. Primary metabolites include sugars, lipids, and fatty acids, whereas secondary metabolites include polyphenols, terpenoids and flavonoids [24,25,17]. The secondary metabolites and other chemical constituents are often attributed to the medicinal value of plants [18]. Phytochemicals (notably secondary metabolites) are bioactive compounds in plants with disease-fighting properties [13,16). Reactive oxygen species (ROS) or free radicals can cause tissue damage through oxidative stress, which can result in various diseases and ageing [6,27]. Antioxidants help minimize or prevent the harmful effects of reactive oxygen species [33].

Sickle cell disease (SCD) is a hereditary disorder resulting from a point mutation in the hemoglobin subunit βetagene on chromosome 11 that encodes the β-globin chain [19]. The point mutationcauses an abnormal form of red blood cells called hemoglobin S (HbS), which contains a single amino acid substitution in the beta-globin chain [34]. In this mutation, one amino acid substitution occurs at the sixth codon (GAG) of the hemoglobinβ-subunit gene, with the amino acid Adenine now replaced by Thymine (GTG). When this occurs,the amino acid Valine now replaces the glutamic acid at this position disrupting normal hemoglobin function[5]. Under low oxygen conditions, the abnormal HbS molecule forms a hydrophobic contact between valine on one chain and other hydrophobic amino acid residues such as alanine, phenylalanine, and leucine among others, which subsequently polymerize and form stiff, rod-like fibres [15]. This tendency to aggregate and form fibres is responsible for the sickling of red blood cells and the resulting blockage of blood vessels, leading to a variety of complications, such as vaso-occlusion, endothelial dysfunction and inflammation [31,27]. These findings suggest that oxyhemoglobin does not trigger the sickling process but rather deoxyhemoglobin, which stimulates the process [11,14].

The sickling of red blood cells results from several factors, including low affinity of HbS to oxygen, physiologically high 2,3-diphosphoglycerate, and increased sphingokinase-1 activity, causing deoxygenation and polymerization of HbS [20]. Cold temperatures increase the tendency of HbS molecules to aggregate and stick together, leading to a higher risk of sickling and vaso-occlusive crises [10]. Therefore, individuals with SCD should dress warmly during cold weather to reduce the occurrence of crises. Research shows that individuals with higher fetalhemoglobin (HbF) levels experience fewer sickling episodes because HbF interferes with the polymerization of sickle red blood cells during the sickling process and is inversely proportional to the severity of sickle cell disease [4, 28]. Therefore, individuals with low HbF levels are at greater risk of mortality.

Though *Z. portoricensis* (a proven antioxidant) is extensively used in traditional medicine to manage many ailments,there is little to no knowledge on its anti-sickling potential [23]. Therefore, this study aims to investigate the anti-sickling potential and phytochemical properties of *Z. portoricensis*.

MATERIALS

Plant Material

Fresh*Z.portoricensis* (Jacq.) H.M.Hern. The stem bark was obtained from the University of Nigeria community in Nsukka, Enugu State, Nigeria. The plant sample was air-dried at room temperature and blended using a mechanical grinder before use. All plant materials were collected and utilized following the standards outlined in the international, national, and institutional rules concerning biodiversity rights. A voucher specimen (No. UNN/11798) was deposited at the University of Nigeria Plant Science and Biotechnology Herbarium Unit for reference.

Instruments and Equipment

Sephadex G25 (Merck, Germany), Centrifuge (Jiangsu Jinyi, China), Spectrumlab 23_A Spectrophotometer (Zhejiang Nade, China), Electronic Scale (G&G, China), Light Microscope (Motic, China), pH meter (Polycase, USA), HH-S4 Water Bath (Searchtech, UK), BUCK M910 Gas Chromatography. Pasteur Pipette, Micropipette, Electrophoresis Paper, Microscope Slides, Microscope Coverslips, Test Tubes, EDTA Bottles, Filter Paper, Hand Gloves, Syringes, Plain Vacutainers, and Glass Chromatography Columns were purchased from a chemical store in the town market in Nsukka.

Reagents

Potassium Phosphate Buffer (10 mM/0.01 M), Ethanol, Distilled Water, Sodium Metabisulfite, and Sodium Chloride were purchased from a chemical store in Ogige market in Nsukka Local Government Area of Enugu State.

Methodology

Preparation of Ethanol Plant Extract

The ethanol plant extract (*Z. portoricensis*stem bark) was prepared following Cyril-Olutayo et al,[9]. The dried powdered plant sample (100 g) was extracted by macerating in 600 ml absolute ethanol for 72 h. The extract was filtered, evaporated to dryness in an oven at 40 °C and kept in the refrigerator for the assays. Different concentrations viz: 1000, 500, and 250 µg/ml were prepared and used for the anti-sickling study.

Extracting Phytochemicals/Botanical compounds

Phytochemical extraction was performed as done previously [7]. The sample was solubilised in 1000µl of pyridine, of which 200µl was transferred to a vial for analysis.

Analysis of Phytochemicals by Gas Chromatography-Flame Ionization Detector

The analysis of phytochemicals was performed on a BUCK M910 Gas chromatography equipped with a flame ionisation detector. A syringe was used to draw 0.1 ml of the extract and injected into the gas chromatography GC machine with FID. Upon sample separation in the GC column, each analyte passes through a flame, fuelled by hydrogen and no air, which ionises the carbon atoms.

Blood Collection

The blood collection was done following Cyril-Olutayo et al [9]. Venous blood (AA and SS) was collected from willing human participants (with informed consent) using sterile techniques. Both samples were quickly placed in Ethylenediaminetetraacetic acid (EDTA) anticoagulant containingtubes which prevent clotting and preserve the blood samples' integrity. They were then stored in a cooling flask with ice.

Preparation of 10 mM Potassium Phosphate Buffer pH 7.8

The potassium phosphate buffer was prepared according [30] with modifications. The anhydrous dipotassium hydrogen phosphate (K_2HPO_4) has a molecular weight (Mw) of 174.18 g. To prepare a 10 mM (0.01 M) potassium phosphate buffer, a precise amount of 0.8709 g K_2HPO_4 was weighed and dissolved in 500 ml of distilled water. The pH level of the solution was then meticulously adjusted to 7.8 using sodium hydroxide while continuously monitoring it with a pH meter.

Preparation of Sephadex G25

Preparation of Sephadex G25 was done according to the method described by Geller et al [12]. with modifications. Sephadex G25, weighing ten grams, was hydrated by soaking it in 1000 ml of distilled water. Next, the mixture was gently boiled at 50 °C for 30 minutes, with regular stirring every 3 minutes to ensure complete hydration. After boiling, the mixture was left to cool, and the distilled water was decanted. Next, 500 ml of potassium phosphate buffer was added to the mixture and allowed to settle for 24 hours. Finally, the mixture was carefully transferred to a column for gel exclusion chromatography.

Equilibration of the Chromatography Column

Equilibration of the Chromatography Column was done according to McCalley [21], with some modifications. To assemble a 50 ml column, the entry point was blocked using glass wool and secured with a glass rod to pin down the glass wool. Sephadex G25 was then added meticulously, ensuring no air bubbles were present. The glass rod was gradually removed after pouring the Sephadex G25. The column was equilibrated by slowly adding potassium phosphate buffer (10 mM, pH 7.8) for 30 minutes until the pH reached 7.8. Throughout the experiment, the column flow rate was kept constant at 15 drops per minute. Using a slower flow rate is advisable to achieve a uniform flow rate. As a preventive measure, a clip was carefully positioned at the column outlet to ensure the potassium buffer solution did not escape the column.

Washing Blood Cells with 1% Normal Saline

Washing of Blood Cells was performed according to Cardigan and colleagues [8] with modifications. To maintain the proper function and purity of blood cells, they were meticulously washed thrice using a 1% normal saline solution, which is an isotonic solution. This isotonic solution was created by dissolving 5 g NaCl in 500 ml of distilled water to prevent potential hemolysis. The solution was then thoroughly mixed by shaking, and two 5 ml AA blood samples were washed with 5 ml saline each in 10 ml centrifuge tubes. Additionally, 2 ml SS blood was washed with 2 ml saline in 10 ml centrifuge tubes. During each wash, a laboratory centrifuge was used to spin the samples at 4000 rpm for 10 minutes.

Removal of Serum

After centrifugation, the supernatant i.e. serum, was extracted precisely using a Pasteur pipette and micropipette. This washing procedure was repeated three times to ensure the complete removal of plasma components. Once the serum was extracted, the red blood cell volume decreased, and the solution was doubled in volume with 1% normal saline. Also, washing was carried out until the third time.

Isolation of Red Blood Cells

Following the third wash, the two centrifuge tubes contained 2 ml of washed red blood cells, which were then lysed using distilled water. The resulting solution from the third wash was doubled in volume with 2 ml of distilled water, and the reagents were gently swirled before being left to stand for an hour at room temperature. The mixture was spun at 4000 rpm for 30 minutes using a laboratory centrifuge. Unfortunately, no distinct layers were observed in the tubes after centrifugation, so the process was repeated twice, each adding 2 ml of distilled water. After the third centrifugation, no distinct layers were visible, so 1 ml of distilled water was added to the 8 ml mixture, and the hemolysis process was repeated. Finally, after centrifugation, two distinct layers could be seen. The supernatant, the hemolysate, was transferred to the EDTA tube, while the pellet containing the plasma membrane, glucose 6-phosphate dehydrogenase, and other enzymes used for metabolism was discarded.

Gel Exclusion Chromatography

The clip at the column outlet was removed to allow the potassium phosphate buffer solution to settle at the top of the gel. Then, using a Pasteur pipette, the hemolysate solution was gently and uniformly introduced into the column while rotating around the circumference to ensure a smooth flow. As the hemolysate progressed, the colours shifted from dark to light red. The column elute was collected at 2.5 ml per vacutainer labelled from 0 to 8. This collected elute contains the hemoglobin (oxyhemoglobin), with tube 0 reserved for UV-visible spectroscopy. Gel exclusion chromatography's principle showed that larger molecules moved faster than smaller ones, and since hemoglobin has a molecular weight of 64,000 daltons, it moves quickly out of the gel beads. Finally, the collected elute was stored with ice in a cooling flask to preserve its quality.

Microscopy of *Z.portoricensis* Stem Bark and Leaves on SS Red Blood Cells

The microscopy procedures were conducted based on methods described previously [9,22]. After centrifugation, the SS red blood cells were washed to prepare the blood sample slides.

A drop of 2% sodium metabisulfite and SS red blood cells were added to a microscope slide and covered with a cover slip to serve as the control. To examine the anti-sickling potential of *Zapotecaportoricensis* stem bark, a drop of 2% sodium metabisulphite and SS red blood cells were added to a drop of the different doses of the plant extract (1000 µg/ml, 500 µg/ml, and 250 µg/ml) on the slide and covered with a cover slip. The red blood cells were counted using a bright field microscope at x40 lens and x10 eyepiece magnification.

The percentage of sickled red blood cells was calculated as follows:

$$\%\ Sickled\ Cells = \frac{Number\ of\ Sickled\ Cells}{Total\ Number\ of\ Cells} \times \frac{100}{1}$$

Finally, the percentage of reverse sickling was calculated as follows:

$$\%\ Reverse\ Sickling = \frac{\%\ Sickled\ Cells\ of\ Control - \%\ Sickled\ Cells\ of\ Sample}{\%\ Sickled\ Cells\ of\ Control} \times \frac{100}{1}$$

RESULTS AND DISCUSSION

Phytochemical Composition of *Zapotecaportoricensis*

Phytochemical analysis of *Z.portoricensis* stem bark revealed the presence of several bioactive compounds, including chalcones, flavonoids, isoflavonoids, polyphenols, phenolic acids, and a cyanogenic glycoside. Notable compounds identified in the stem bark include butein, genistein, daidzein, epicatechin, and ellagic acid, which have been previously associated with anti-sickling and antioxidant properties in the literature. These findings suggest that the phytochemical diversity in *Z. portoricensis* may contribute to its therapeutic potential (Table 1).

Anti-Sickling Activity

The anti-sickling potential of *Z. portoricensis* was evaluated by treating SS red blood cells with 2% sodium metabisulfite to induce sickling. The stem bark extract demonstrated significant anti-sickling activity, achieving 85% reverse sickling at a concentration of 250 µg/ml within 10 minutes, as observed microscopically (Figure 3). Higher concentrations (500 and 1000 µg/ml) also demonstrated substantial reverse sickling activity but were less effective than the 250 µg/ml concentration.

The dose-dependent anti-sickling effect observed in the extract highlights the efficacy of the stem bark, is likely due to the higher concentration of bioactive compounds such as butein and ellagic acid. These compounds are known to interact with hemoglobin and the plasma membrane, preventing or reversing the sickling process.

Microscopic Observations

Microscopic examination further confirmed the anti-sickling activity of *Z. portoricensis* extracts. In untreated samples, 90.74% of SS red blood cells were sickled. Treatment with the stem bark extract at 250 µg/ml reduced the proportion of SS cells to 14.69% (Figure 3). These observations highlight the efficacy of the stem bark extract in reversing cell sickling.

Comparative Analysis

Results in this study are in agreement with research demonstrating the anti-sickling and antioxidant properties of aromatic compounds with hydroxyl groups. Notable compounds identified in *Z. portoricensis* stem bark, including quercetin, vanillic acid, and naringenin, have been shown to possess outstanding anti-sickling activity.

Additionally, the antioxidant properties of flavonoids and phenolic acids in the extracts likely contribute to their therapeutic potential by preventing hemolysis and preserving red blood cell integrity.

Conclusion

This study demonstrates that *Z. portoricensis* stem bark possesses significant anti-sickling and antioxidant activities. The results indicate that *Z. portoricensis*has natural therapeutic properties that make it a feasible alternative therapy for managing sickle cell disease. Further work is required to assess what unique bioactive compounds promote reversal of SS cell shape in people with the SS disease. It will also be beneficially effective to evaluate the efficacy of these botanical compounds *in vivo*.

Table 1: Phytochemical Components of the Ethanol Extract of Zapotecaportoricensis Stem Bark

Ret Time [min]	Area [pA*s]	Amt/Area	Amount [ppm]	Name
3.692	1.22597	1.33879e-1	1.64133e-1	Catechin
4.267	1366.32178	1.40422e-1	191.86199	Butein
5.176	19.69657	1.40131e-1	2.76011	Quercetin
6.358	79.55717	1.40476e-1	11.17590	Luteolin
7.405	15.36688	1.40064e-1	2.15235	Artemetin
8.287	8.48381	1.39549e-1	1.18391	Retusin
9.283	91.03340	1.40499e-1	12.79011	Ellagic acid
10.009	31.40797	1.40311e-1	4.40689	Vanillic acid
10.680	1.38894	1.33965e-1	1.86069e-1	Naringenin
13.007	2.55276	1.36693e-1	3.48945e-1	Myricetin
13.444	182.52982	1.40555e-1	25.65547	Epicatechin
14.061	205.02066	1.40594e-1	28.82462	Daidzein
14.723	284.40250	1.40132e-1	39.85385	Genistein
15.064	43.12519	1.40343e-1	6.05232	Apigenin
15.730	1.69993	1.34219e-1	2.28162e-1	Linamarin
Total:			**331.32744**	

Table 2: Purity and Concentration of the AA Blood Hemoglobin at Different Time Intervals

Sample	Time (mins)	Hemoglobin Purity	Hemoglobin Concentration (mM)
Hb Only (Control)	0	1.0254	0.1197
Hb + 1000 µg/ml Z. portoricensis Stem Bark	0	1.0045	0.0660
Hb + 500 µg/ml Z. portoricensis Stem Bark	0	1.0275	0.0610
Hb + 250 µg/ml Z. portoricensis Stem Bark	0	1.0320	0.0611
Hb + 1000 µg/ml Z. portoricensis Stem Bark	10	1.0017	0.0659
Hb + 500 µg/ml Z. portoricensis Stem Bark	10	1.0273	0.0604
Hb + 250 µg/ml Z. portoricensis Stem Bark	10	1.0297	0.0608
Hb + 1000 µg/ml Z. portoricensis Stem Bark	20	0.9991	0.0653
Hb + 500 µg/ml Z. portoricensis Stem Bark	20	1.0249	0.0600
Hb + 250 µg/ml Z. portoricensis Stem Bark	20	1.0278	0.0597

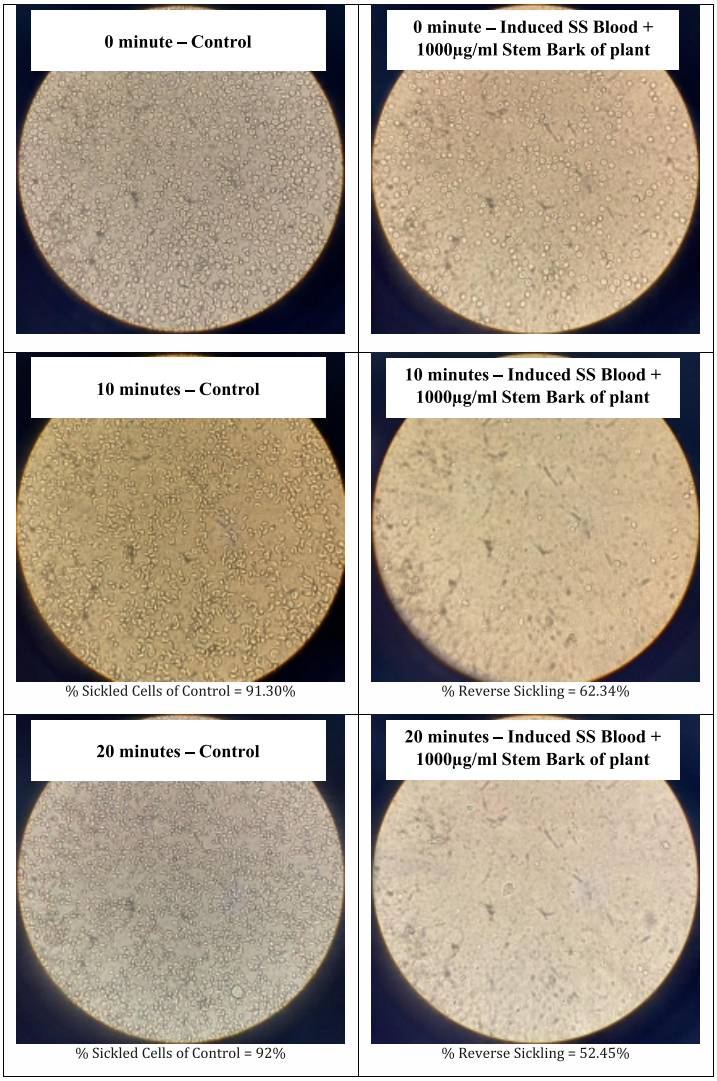
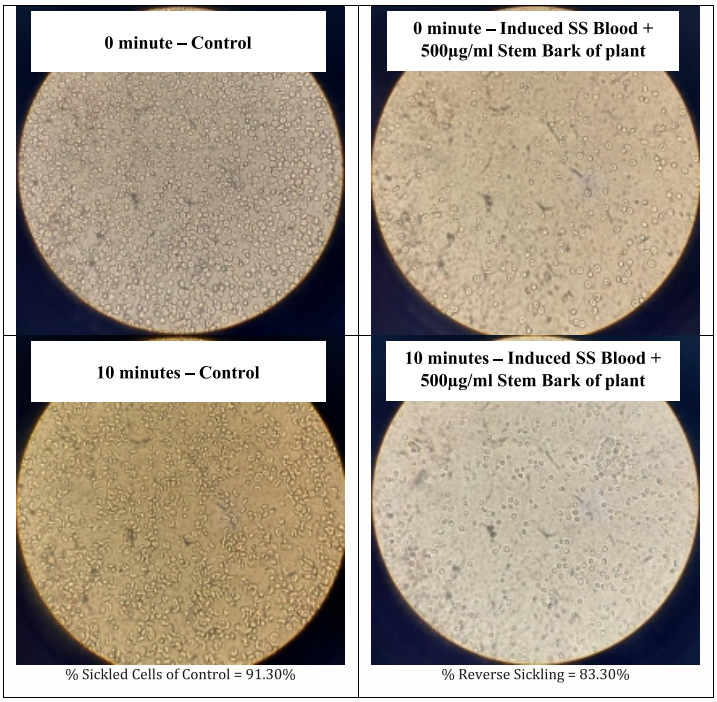


Figure 1: Microscopic Anti-Sickling Potential of 1000 µg/ml Zapotecaportoricensis Stem Bark



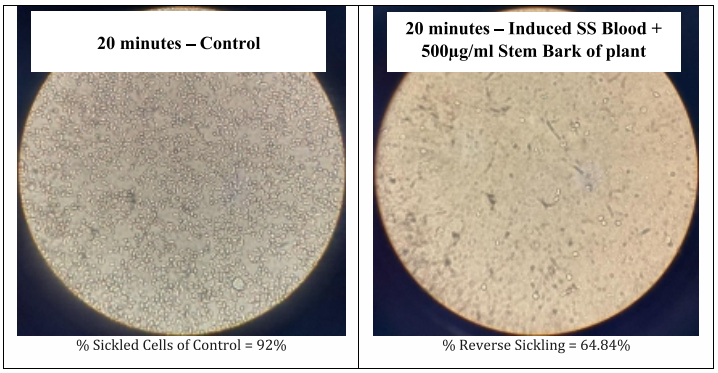


Figure 2: Microscopic Anti-Sickling Potential of 500 µg/ml Zapotecaportoricensis Stem Bark

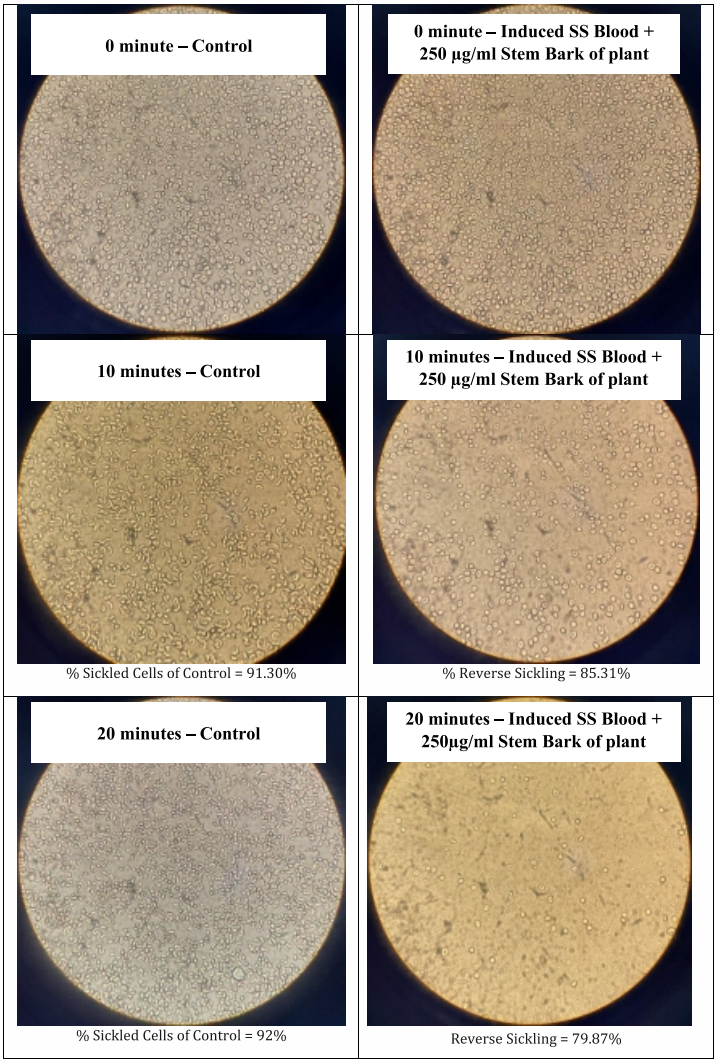


Figure 3: Microscopic Anti-Sickling Potential of 250 µg/ml Zapotecaportoricensis Stem Bark

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