Evaluation of Antisickling Activity and Antibacterial Potential of Methanolic Root Extract of *Ximenia americana* Linn. (*Olacacaea*)

Abstract

Aims: This research work was done in order to evaluate *in vitro*antisickling activity, and antibacterial potentials of the methanolic root extract of *Ximenia americana*, Linn. *Olacaceae*.

Study Design: It is an experimental research work.

Place and Duration of Study: Department of Microbiology and Biotechnology, National Institute of Pharmaceutical Research and Development (NIPRD), Idu, Abuja, Nigeria.

Methodology: Evaluation of the antisickling activity of the methanolic root extract of *Ximenia americana* was carried out in two-fold; viz. the ability of the plant extract to inhibit or prevent sickling (antisickling effect) of erythrocytes from blood samples obtained from sickle cell disease (SCD) volunteers on one hand, and the ability of the extract to reversed pre-sickled erythrocytes using standard protocols. The antibacterial assay was done using microtiter broth dilution method to determine the minimum inhibitory concentration (MIC) on some selected encapsulated bacterial (*E. coli, Salmonella typhi, Staphylococcus aureus, Pseudomonas mirabilis, Pseudomonasaeruginosa, Bacillus subtilis,* and *Klebsiella pneumonia*) that have been reported to caused complications in SCD.

Results: The methanolic root extract of *Ximenia americana* showed a concentration dependent antisickling activity across concentration gradient. The percent sickling inhibition was dose-dependent (0.05 mg = 60 %, 0.5 mg = 62 %, 1 mg = 88 %, 2 mg = 87 %, and 4 mg = 90 %), this result was statistically significant ($P< 0.05$ Vs control) when compared to the untreated group (25 %). Similarly, the result of the sickle red cell reversal by the test extract demonstrated a percent dose-dependent reversibility (0.05 mg = 36 %, 0.5 mg = 40 %, 1 mg = 48 %, 2 mg =

58%, and 4 mg = 63 %), a result that was significantly (*P*< 0.5 vs control) compared to the control (19 %). The antimicrobial assay result showed that, the sensitivity of the selected microorganisms to the plant extract was concentration dependent. Staphylococcus aureus and Bacillus subtilis showed better sensitivity to the extract with higher zones of inhibition (8 mm and 7 mm) at 80 mg/mL concentration.

Conclusion: Methanolic root extract of *Ximenia americana* L demonstrated a significant dose-dependence antisicklingand sickle cell reversal activities, with *P*< 0.05 vscontrol. Also, the extract showed sensitivity against some selected bacteria isolates as a result possess antibacterial activity against susceptible microorganism.

Key words:Antisickling; Sickle cell reversal; Antibacterial; *Ximenia americana*; Assay.

1. INTRODUCTION

Sickle cell disease (SCD), a neglected genetic disorder [1,2] is transmitted from parents to offspring [3 - 5]. According to experts, the disease is classified as a major public health challenge of the century and is characterized by a high morbidity and mortality among low- and middle-income countries, particularly in developing countries of the world where facilities, and economic powers necessary to managing the disease are in short supply [6 - 10],[1],[11], [5]. The disease affects and damage multiple organs in the body of patients [1], [11-13].

Sickle cell disease, a term that encompasses mutations of the β-globin gene [14[, [15], [5], [12], [16] is believed to originate from among people of Africa ancestor, especially in the Sub-Saharan region [17],

[18], [19], [20], [21]. However, it is now well distributed in almost all the continent of the world [22], thus, assuming a global proportion.

It is estimated that about 7% of human population in the world are carriers of the sickle cell gene [22, 23], and 300,000 neonates are estimated to be born with SCD yearly [17], [12], [5]. Earlier research findings on SCD showed that an average of 100,000 Americans is affected with the disease, and it is believed that there are a million of other sufferers of the disease around the world [15]. The disease is reported to have a high prevalent in Africa, Central and South America, Asian (particularly India), and the Mediterranean region [24, 25].

The genetic challenge associated with sickle cell disease involve the substitution of a single deoxyribonucleic acid (DNA) nucleotide in the sixth codon of the gene that synthesis β-hemoglobin [26], [23]. The resultant hemoglobinopathy depends on the type, shape and position of the amino acid substitution that takes placed. Hence, this gives the different classification as while as severity of the disease. Thus, substitution of a flexible hydrophilic (polar) glutamic acid (Glu) residue with a non-flexible hydrophobic (non-polar) valine (Val) residue at the position sixth in the β-globin chain results in formation of the most severe form of SCD sometime referred to as sickle cell anemia (Hb SS), this is the serious form of the disease, and is typified by frequent decrease in hemoglobin level or anemia [26], [28-30]. Also, substitution of the amino acid glutamic acid with lysine at position sixth of the β-globin chain result in formation of a hemoglobin variant with a sickling disorder known as Hb SC which often is occasion with severe symptoms. Similarly, substitution of Glu with glutamine at position 121 of the β-globin chain result in formation of sickle cell disorder Hb SD. Symptoms of this form of the disease can be moderate to severe when compared to Hb SS, and this depend on other under-lining disease conditions [31], [15],[23]. Also, when the amino acid lysine is substituted for glutamic acid at position 26^{th} of the β-globin, a hemoglobin variant Hb SE is form, this form of SCD has mild to moderate symptoms when compare to Hb SS. In any case, these substitutions do results in red blood cells that are mechanically fragile, poorly deformed, and hence causing impairment to free flow of blood, and may cause other pathophysiological conditions such as, polymerization, red blood cell sickling (under reduced oxygen tension), anemia (due

to frequent hemolysis of sickle red blood cell), vaso-occlusion or blockage of blood vessels among other numerous problems that are associated with the disease [32-34], [9], [26].

One fundamental challenge and or complication of sickle cell disease is its association in pregnancy. This often constitutes a high risk to both the mother and the unborn fetus. Thus, resulting in unwanted obstetric complications to mothers, premature delivery and low birth weight to infant, with attendance increase morbidity and mortality in both cases [36- 38], [14].

SCD has been identified, and classified as a genetic disease. Hence, there is yet to be a specific medication(s) for cure. However, there are many management approaches (both Pharmacologic and Non-Pharmacologic) that are employed in order to mitigate the signs and symptoms of the disease among they poor majority in most developing nations including Nigeria (Com). The major goal for management is to prevent disease progression and injury, thereby promoting healthy living so as to prolonging lives of those with the disease [39, 40].

Some measures employed to achieve this goal include, but not limited to the following, the use of hydroxyurea, blood transfusion, and bone marrow or hematopoietic stem-cell transplant [41-44]. Some of these approaches are often expensive and the few clinically available medications are toxic to the patients, and others (bone marrow or hematopoietic stem-cell transplant) are associated with high risk, require expertise, and allogeneic- match sibling as donors, conditions that are not easily met by the poor patients [45-47]. Because of these, it became imperative to look for alternative means of treatment for the disease that is seemly cheap, effective, and believed to have less adverse effects. Hence, the need to explore natural remedy such as the use of medicinal plants [49], [46]. Many of these medicinal plants have been demonstrated to possess useful antisickling activity. They include, *Carica papaya, Cajanus cajan, terminalia catapa, Zanthoxyllumzanthoxyloides, Gardenia ternifolia, Jovis-tonantis, Uapacaheudelotii*,among others [46], [49-51]. This is also true with the plant *Ximenia americana*Linn. *Olacaceae*, a recipe which have been anecdotally used with acclaimed success for managing patients with sickle cell diseases by a traditional medical healer in Kpadock clank among the Kagoro (Gworok) community in the southern part of Kaduna State, Nigeria.

Ximenia americana is one of the eight species of *Olacaceae*family [52]. The plant grows naturally in tropical and temperate regions of the world [53], and has been reported to possess valuable traditional medical uses [52], [54-58]' some of these ethnomedical uses have been well documented in literature. However, this is the first report on the traditional use of this plant (*Ximenia americana*) in managing sickle cell disease.

2. Materials and Method

2.1Plant material collection, identification and authentication

Ximenia americana was collected, identified, and authenticated according to [59].

2.2Informed Consent, and Ethical Approval

Ethical clearance was obtained from the Health Research Ethics Committee (HREC) of National Institute of Pharmaceutical Research and Development (NIPRD) Idu, Abuja(CC/PF527).

Blood samples were collected from donors (confirmed sickle cell patients), who had read and sign informed consent form every week at the sickle cell clinic of the National Institute of Pharmaceutical Research and Development (NIPRD) Idu, Abuja, and the collected samples were handled in accordance to the standards laid down in 1964 Helsinki Declaration [60].

2.3Blood Sample Collection, and Preparation

5 mL fresh blood was collected from antecubital vein of confirmed, stabled sickle cell patients (volunteers) who are not in crises, and had not received normal blood (Hb AA) transfusion at least 30 days prior to the time of recruitment. Participants enrolled for the study were children and adult of between 5 and 37 years of age, and of both gender (male and female). The blood samples were collected into cleaned apathogenic sodium ethylene diamine tetra acetic acid (EDTA) bottles for the research work.

2.4Sample Size Determination

Minimum sample size in this research was determined using a formular (stated below) previously described by [61], and reported by [62]:

$$n = \frac{Z^2 pq}{d^2} \ldots\ldots\ldots Equation\ 1$$

Where;

n = minimum sample size

Z = value of normal curve corresponding to 95% confidence interval (1.96)

P = prevalence of sickle cell disease (2.3%)

q = 1- *P* (1- 0.023 = 0.977)

d = error margin or level of significance (5%)

$$n = \frac{\mathbf{1.96^2 \times 0.023 \times 0.977}}{\mathbf{0.05^2}}$$

= 34.5

$$\cong 35$$

2.5 Antisickling Activity of Methanolic Root Extract of X. americana

Hb SS blood hemolysate (0.2 mL) was pipetted into test tubes (in triplicates); 0.2 mL phosphate buffered saline solution (pH 7.4) and 0.2 mL of the extracts were added. The mixture was overlaid with liquid paraffin (1 mL) and incubated in a thermostatic water bath at 37°C for 4 hr. Thereafter, freshly prepared 2% w/v sodium metabisulphite ($Na_2O_2S_5$) solution (0.6 mL) was carefully added under the liquid paraffin layer and incubated for 4 h period. The mixture was thoroughly and carefully mixed by rolling the test tubes between the palm of the hands. The final mixture was incubated further for another 1h 30 m at 37°C in a water bath. Then, the liquid paraffin layer was carefully removed with a Pasteur pipette and the resultant mixture was fixed in 3 mL of 5% v/v buffered formalin. The experiment was set up in triplicate in synchronicity with negative control (where 0.2 mL of 0.9%w/v NaCl isotonic saline/normal saline solution was used to replace the plant extract). The percent inhibitory (Equation 2) activity for each concentration of the extract (0.05, 0.5, 1, 2, and 4 mg/mL) was calculated, the results obtained were presented as mean standard error of mean for the individual triplicates.

$$Percent\ (\%) Inhibition\ of\ Polymerisation = \frac{Number\ of\ Sickled\ Cells}{Total\ Number\ of\ Cells\ counted} \times 100 \ldots\ldots\ldots\ldots Equation\ 2$$

2.6 Evaluation of Sickled Reversal Activity of Methanolic Root Extract of X. americana

Hb SS blood hemolysate (0.2 mL) was placed in a test tube (in triplicate), 0.2 mL phosphate buffered solution was added and the mixture was overlaid with 1 mL liquid paraffin. Thereafter, 0.6 mL 2% w/v sodium metabisulfite ($Na_2S_2O_5$) solution (reductant or deoxygenating agent) was introduced gently under the liquid paraffin layer. The mixture was thoroughly and carefully mixed by rolling the test tube between the palm of the hands before incubating at 37°C in a thermostatic water bath for 1h 30 m. Then, 0.2 mL of the plant extracts was carefully added under the liquid paraffin layer and incubated further for 6 h. The experiment was set up in triplicates with a negative control, where 0.9% w/v NaCl (normal saline) solution was used in place of the extracts. After incubation, the liquid paraffin layer was carefully removed with Pasteur pipette and 3 mL of 5% v/v buffered formalin solution was added. The mixture was thoroughly mixed to ensure proper fixation [63]. The percentage reversal activity for each sample extract was calculated (Equation 3) from the results appropriately, and presented as mean standard error of mean for the individual triplicates. This was done for all set-up of the test extract and the experimental control.

The percentage reversal of polymerization of the test extract was calculated using the formula:

$$Percent\ (\%) Reversal\ of\ Activity = \frac{Number\ of\ sickled\ cells}{Total\ number\ of\ cells\ counted} \times 100 \ldots\ldots\ldots Equation\ 3$$

2.7 Cell(s) counting

Slides were prepared from fixed cells after centrifugation. The fixed cell mixtures were each centrifuged and their supernatants decanted. With the use of a capillary tube, two drops were applied on a cleaned dried microscope slide, carefully covered with a cover slip and with the use of a high-power objective (x 100) of the microscope, 400 cells (both sickled and non-sickled erythrocytes) were counted and the percent sickled cells estimated (as in equations 2 and 3 above).

2.8 Microbiological Assay

The activity of the methanolic root extract of *Ximenia americana* was challenged using some selected micro-organism that are known to cause complications in sickle cell diseases. Literatures has identified infections by micro- organisms (encapsulated bacteria) as one of the leading causes of morbidity and death among these categories of patients particularly children [64- 67]. The most implicated bacteria in this disease condition include; *Streptococcus pneumoniae, Escherichia coli, Hemophilus influenzae, Neisseria meningitides, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Salmonellatyphi* that often leads to osteomyelitis [64, 65], [69].

These infectious organisms do precipitates sickle cell crisis that can be life threatening and often times cause excruciating pains. Therefore, infections prevention, management, and or treatment are important cornerstone of comprehensive health care in SCD [69], [71, 72].

2.9 Methodology for Antimicrobial Assay of Methanolic Root Extract of X. americana

2.9.1 Microbiological media used for the assay

The media used for this study include Mueller Hinton Agar, Mueller Hinton Broth, Peptone water. All the media were prepared according to the manufacturer's instruction.

2.9.2 Preparation of inocula

Stock cultures were maintained on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller- Hinton broth (MHB) for reactivation by culturing overnight at 37^{O}C. Cultures were diluted with fresh MHB

and compared with 0.5 McFarland standard to achieve values approximately $1.5x10^8$ colony forming unit of bacteria cells.

2.10 In-vitro Antimicrobial Susceptibility Assay of the Methanolic Extract

The extract of *Ximenia americana* was first dissolved in dimethyl sulfoxide (DMSO) and then sterile distilled water, to give a starting concentration of 800 mg/mL. The dissolved extract was diluted across the 96-well microlitre plate in a two-fold serial dilution to give final testing concentrations of 80, 40, 20 and 10 mg/mL. The same procedure was repeated for the control drug, Ciprofloxacin [Sigma Aldrich Inc] at a concentration of 10 µg/mL used as positive control drug, and extracts/drug free medium with culture suspensions were used as negative control. Each extract concentration was assayed in duplicate. The plates were then incubated for 24 hours at 37^OC. After the 24 hours incubation, 25 µL of tetrazolium salt dye was added to all the wells, re-incubated over-night, and observed for absence or presence of microbial growth by colour change in the wells. The MIC was defined as the lowest drug/extract concentration that prevented the colour change of the tetrazolium dye to pink. Colourless well was interpreted as there is no bacterial growth and pink colour was interpreted as growth occurrence.

2.11 Data Presentation and Statistical Analysis

Data generated from this research work was presented as mean ± SEM of independent biological replicates. Every investigation was carried out in two or three independent experiments. One- way analysis of variance (ANOVA) statistical tool was used for the antimicrobial assay, while two-way ANOVA was used for the antisickling assay. Dunnet*post hoc* test was employed for multiple comparisonbetween groups mean versus control using GraphPad Prism version 8.0.2 (263) for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). ANOVA was taken as statistically significant at $P < 0.05$.

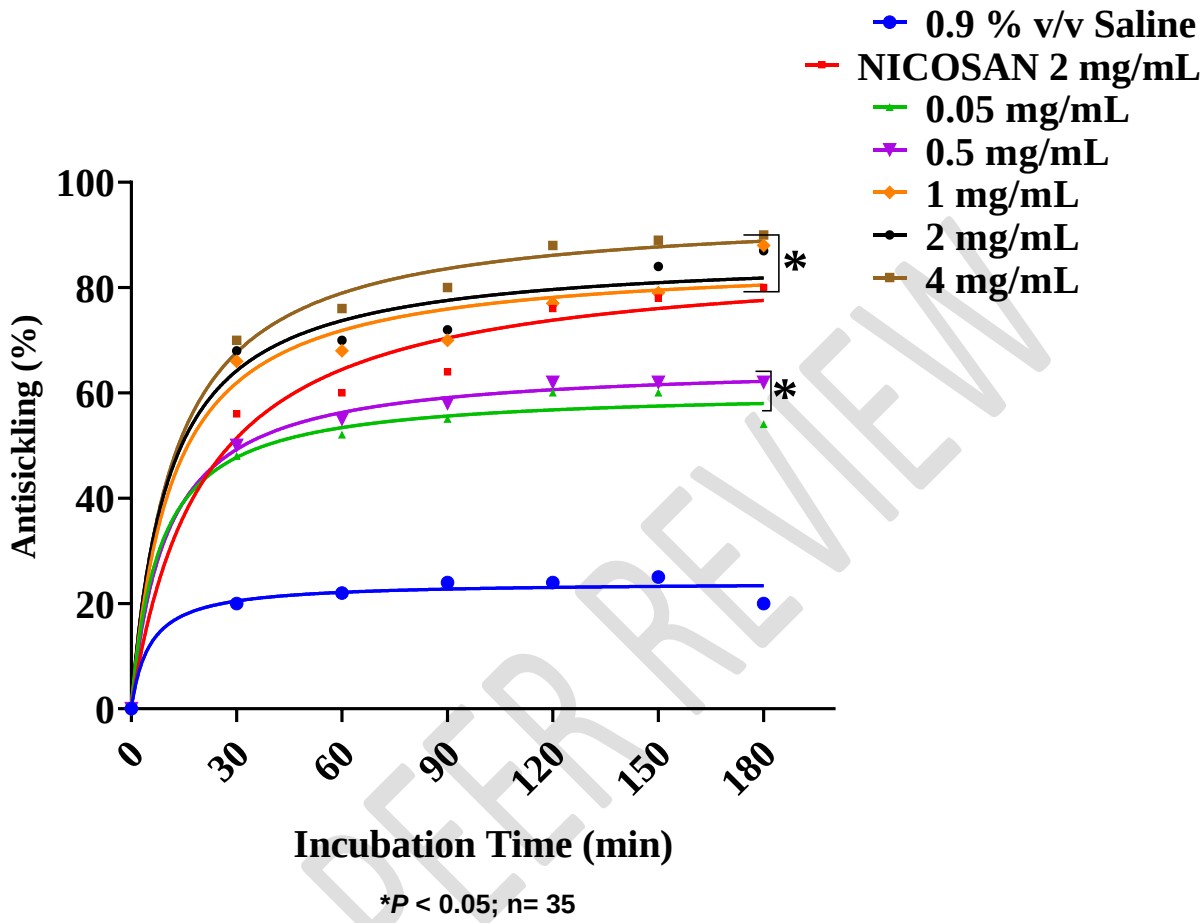
3. Results and discussion

3.1 Powered Ximenia americana methanolic Root Extract

Plate 1. Freeze-dried Methanolic Root Extract of *X. americana*.

The dried powder of methanolic root extract of the plant is dark-brown in color.

3.2 Inhibitory Effect of *X. americana* Methanolic Root Extract on Sickle Red Cell

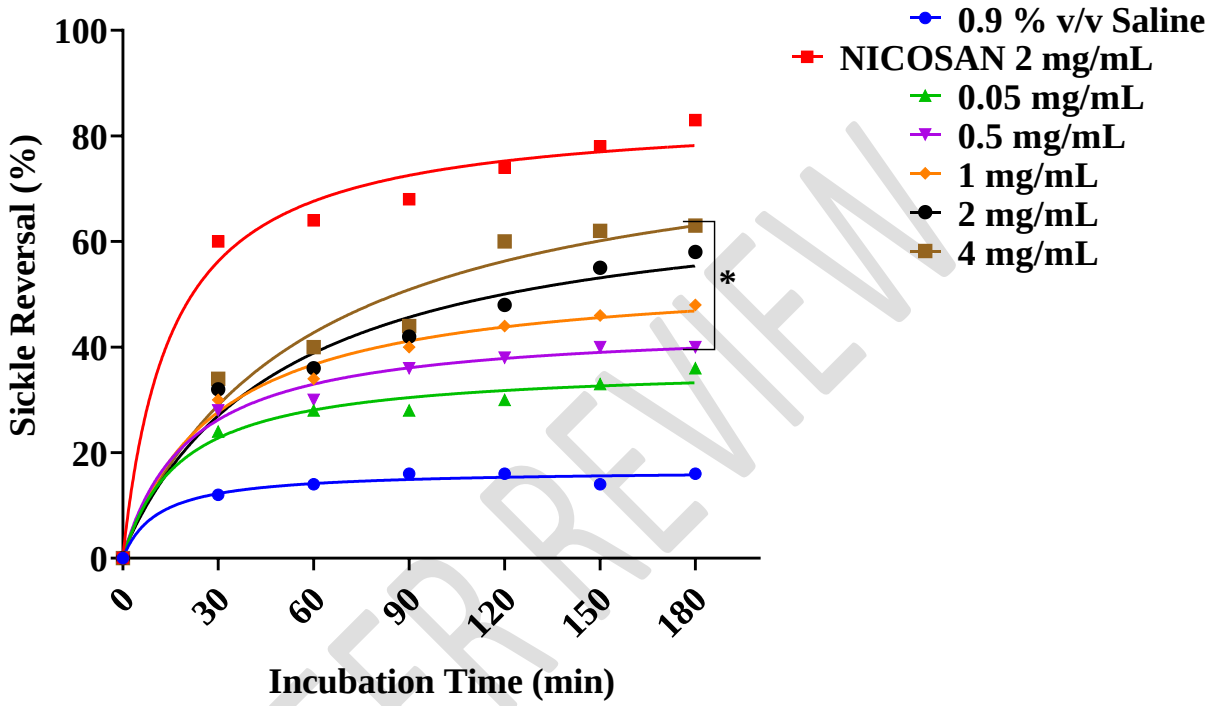


***P* < 0.05; n= 35**

Fig. 1.Anti-sickling Effect of Methanolic Root Extract of *Ximenia americana*

The root extract of *X. americana* significantly (*P* < 0.05 vs control) inhibited sickling induced by $Na_2S_2O_5$*in-vitro* in a dose-dependent manner.

3.3 Sickled Red Cell Reversal Effect of Methanolic Root Extract of *X. americana*



***p* < 0.05; n = 35**

Fig. 2. Sickling Reversal Effect of Methanolic Root Extract of *Ximenia americana*

The methanolic root extract of the plant does significantly (*P*< 0.05 vs control) reversed sickling induced by $Na_2S_2O_5$ *invitro*.

3.4 Anti- Microbial Potentials of *X. americana* Methanolic Root Extract

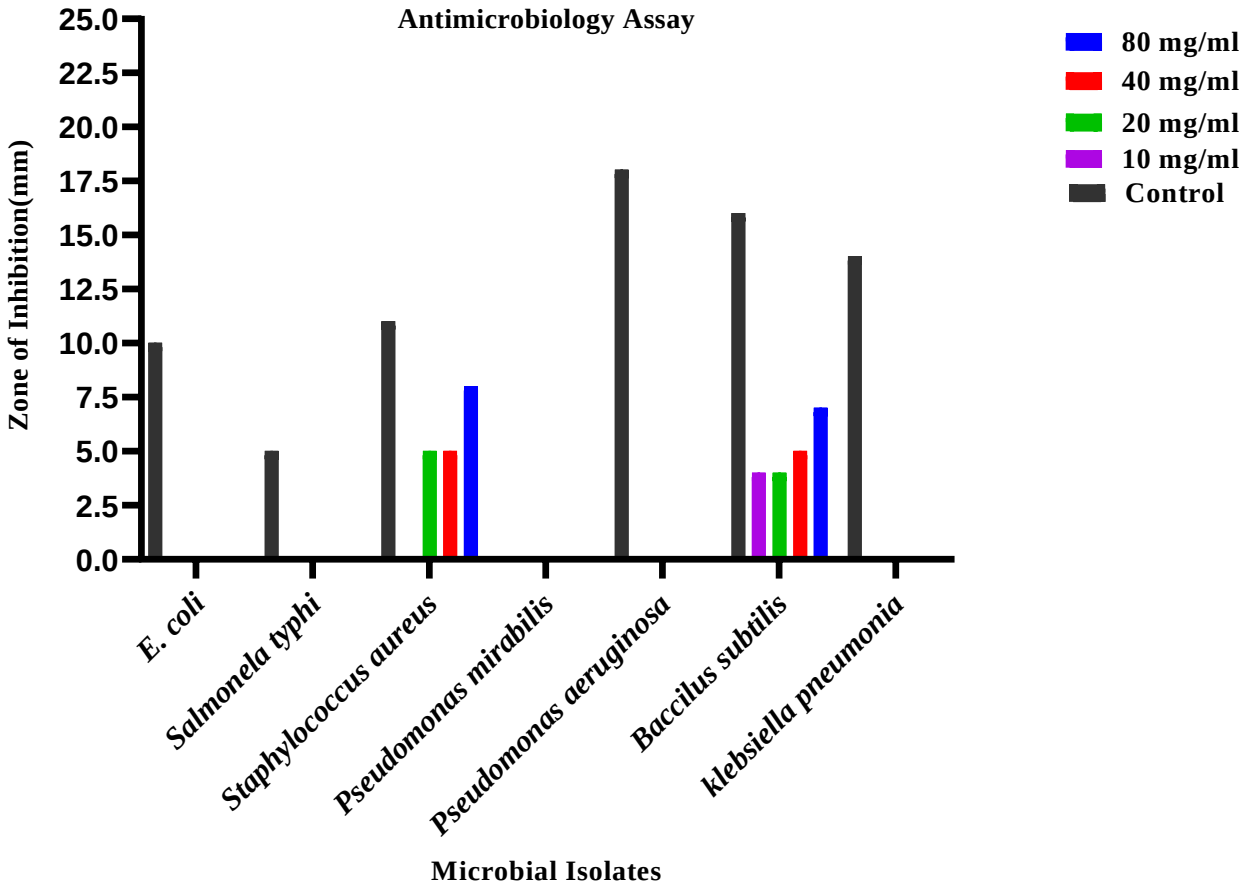


Fig. 3. Antibacterial Effect of *X. americana* Methanol root extract on micro-organism isolates

3.5 Discussion

Sickle cell disease (SCD) is an inherited hemoglobinopathy that is transmitted from parent to offspring. The disease makes the hemoglobin assumed a sickle-shape which at low oxygen tension leads to formation of polymer bundles, resulting in a decrease oxygen carrying capacity of the red cell. This defect does result in crisis manifesting in form of pains at the affected part or organ of the body. Owing to the fact that SCD is a genetic disorder, it has become increasingly difficult to get a cure for the disease. However, there is increasing evidence that the disease can be managed effectively using both orthodox, and traditional medicine (using plant material) approached. Due to paucity of orthodox medicine, it become necessary to look for alternatives, hence the research on the plant *Ximenia americana* Linn.

The freeze-dried powder extract of *X. americana* is dark -brown in colour (Plate 1). The evaluation of antisickling activity of *Ximenia americana* methanolic root extract was carried out in order to determine the ability of the extract to prevent or inhibit sickling of erythrocytes on one hand, and the ability of the extract to reverse sickling in pre-sickled red blood cell on another hand.

From the results obtained (Fig. 1), the plant extract demonstrated an excellent inhibition of sickling with the chosen concentrations. The observed percent sickling inhibition was concentration-dependent (0.05 mg = 60 %, 0.5 mg = 62 %, 1 mg = 88 %, 2 mg = 87 %, and 4 mg = 90 %), this result was significantly different ($P < 0.05$ vs control) when compared to the untreated group (25 %).

Similarly, the result from the sickle cell reversal (Fig. 2) by the test extract demonstrated a percent dose-dependent reversibility (0.05 mg = 36 %, 0.5 mg = 40 %, 1 mg = 48 %, 2 mg 58 %, and 4 mg = 63 %). The result was significant ($P<0.05$ vs control) at 0.5 – 4 mg of extract. Thus, the extract can be said to possess both antisickling andsickled cell reversal potentials *in vitro*.

Similarly, the ability of the methanolic root extract of *Ximenia americana* to inhibit the growth and proliferation of some of the selected pathogenic bacteria isolates (*E. coli, Salmonella typhi, Staphylococcus aureus, Pseudomonas mirabilis, Pseudomonas aeruginosa, Bacillus subtilis, and Klebsiella pneumonia*) was assayed. From the result (Fig. 3), the sensitivity of these organisms to the extract was concentration dependent. *Staphylococcus aureus and Bacillus subtilis* showed better

sensitivity to the extract with higher zones of inhibition (8 mm and 7 mm) at 80 mg/mL concentration. From the chat, it is worth noting that *Bacillus subtilis* was sensitive to all the selected concentrations (80, 40, 20 and 10 mg/mL) with zones of inhibitions of 7, 5, 4, and 4 mm respectively. This result is in complete agreement with [73-75],who reported the sensitivity of the root extract of this plant to *staphylococcus aureus* and *Bacillus subtilis.* These authors in addition reported sensitivity of the plant part to other organism such as *E. coli, Salmonella typhi, Pseudomonas mirabilis, Pseudomonas aeruginosa, and Klebsiella pneumonia*, a result that is at variant with the outcome of our research work. This observed variation could be due to the different strain of microbes used, the phytogeographical location of the plant, and its nutritional status. In addition, the variation could also be due to variability in the extraction methods, and solvent polarity [75, 76]. Similarly, other authors had reported on the, antifungal and antiparasitic activities of this plant part [58], [73].

The authentication of the antimicrobial potentials possess by this plant may be of benefit to SCD patients who may suffer from these infectious bacteria's secondary to other pathophysiology of SCD such as polymerization, vaso-occlusive crises and hemolytic anemia.

4 Conclusion

This research has established with empirical data, the antisickling and antimicrobial potential of *Ximenia americana*. Hence, validating its used in management of sickle cell disease in folk medicine.

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