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*Full Length Research Paper*

# *In vitro* antisickling effects of *Xylopia aethiopica* and *Monodora myristica*

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**The antisickling effects of two indigenous spices *Xylopia aethiopica* and *Monodora myristica* were investigated. Two hundred grams (200g) of each powdered sample was divided into two equal parts. One part was used for crude aqueous extraction (CAE) and the other, for Batch process extraction, with chloroform, methanol, butanol, and water to yield; the fat-soluble (FAS), butanol-soluble (BUS) and water-soluble extracts (WAS) respectively. The FAS, BUS, CAE and WAS fractions exhibited profound antisickling effectiveness by inhibiting HbSS polymerization to varying degrees from 70% for FAS to 90% for CAEs in fifteen (15) min. The CAE and WAS fractions were equally able to improve $Fe^{2+}/Fe^{3+}$ ratio for CAEs and 13 - 100% for WAS fractions respectively. These fractions also reversed already sickled erythrocytes, with the WAS fractions having less time than the CAE fractions. Thin layer chromatographic (TLC) analysis showed that the extracts generally contain some antisickling amino acids such as Arg. Tyr and Asp at varying concentrations. The total free amino acid concentrations of the samples revealed high concentrations of such, with the CAE fractions of *X. aethiopica* and *M. myristica* having concentrations of 1028 and 1680mg/100g of samples respectively. Results suggest that these spices when used in combination with other nutritious regimen like fruits, fish and legumes, may be a promising option for the effective management of sickle cell disease and a gamut of its pathophysiological complications.**

**Key words:** *Xylopia aethiopica, Monodora myristica,* sickle cell disease, $Fe^{2+}/Fe^{3+}$ ratio polymerization

## INTRODUCTION

Sickle cell disease is one of the most prevalent hereditary disorders with prominent morbidity and mortality. While the disease may affect various ethnic groups such as the people of the Hispanic and Middle East descent, it affects those of African descent, the more. The most clinical manifestations are largely due to hemolytic processes leading to severe anemia and vaso-occlusive crises resulting in pain and organ damage (Cotran et al., 1999).

Several therapies have been prognosed and many chemical substances investigated for their possible role in the management of sickle cell disease (SCD). Among the many potential agents employed to prevent or reverse sickling include: Hydroxyurea (HU). Erythropoietin, Tucaresol, Ciklervit™ etc. Although hydroxyurea has been found, to be very effective in many patients, in others, it has yielded many pronounced side effects (Charache, 1995).

In the search for effective chemotherapeutic agents with less adverse effects on sickle cell disease patients, many researchers have shown the antisickling effectiveness of most nutrients derived from plants and animals and these may provide possible and reliable option for the effective management of sickle cell disease (Uzoegwu, 1997; Nwaoguikpe and Uwakwe, 2005). The relationship between SCD and nutrition has been systematically reviewed and documented (Ekeke, 1997). Several investigators have commented on the abnormally low levels of certain micronutrients in sickle cell blood or that certain dietary constituent such as thiocyanate and Ascorbic acid (or micro-nutrients such as zinc) is beneficial in sickle cell disease (Agbai, 1986; Ekeke et al., 2001). Apart from ascorbic acid, other vitamins such as Vitamin E have been found to be beneficial to the sickler.

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Root crops, legumes, fruits and vegetables have been prescribed for sicklers (Uzoegwu, 1995; Ekeke, 1997).

Some ripe fruit juices, edible legumes and free amino acids, have been shown *in vitro* to possess antigelling properties (Ekeke et al., 2001: Nwaoguikpe and Uwakwe, 2005).

Today, spices are used in wines, foods, beverages, cosmetics, tooth pastes and in medicine as adjuvants. Some spices have anti-microbial and soothing properties (Purseglove et al., 1991). A spice being a vegetable substance of indigenous or exotic origin, being aromatic, is used to enhance the flavour of food. They are derived from rhizomes, bark of trees, leaves, fruits, seeds and other parts of a plant (Kochler, 1986): The inhibitory effect of spices oil could be attributed to the presence of an aromatic nucleus containing a polar functional group. *Xylopia aethiopica* has been reported in literature to possess medicinal and nutritional values (Nwachukwu, 2000). Chemical constituents include essential oils, resin, anonaccin, reberoside, avocien, rubersole, alkaloids, tannins, oxalate and flavonoids.

In 1993, Iwu also identified vitamins, A, B, C, D, E, proteins, and minerals (Nwachukwu, 2000). The fruit is used for soup making, particularly for nursing mothers. In folk-medicine, it is used for the treatment of biliousness, bronchitis and dysentery (Iwu, 1993).

*Monodora myristica* is found in tropical Africa. The fruits contain brown-oval seeds which have a mint smell. It is the seed that is used as spice. Chemical constituents are fiberro-latic oils, resin, terpene, lactose, arocine, saponins, flavonoids and tannins (Iwu, 1993).

The aim of the research work is to investigate the possible antisickling effects of these two spices based on some of their chemical constituents. Moreover their ability to improve the $Fe^{2+}/Fe^{3+}$ ratio would be an added advantage in increasing the oxygen affinity of the red blood cells.

## MATERIALS AND METHODS

**Chemicals:** Ninhydri, butanol, sodium metabisulphite and hydrochloric acid were from Sigma Chemical Company, Poole Dorset, UK. Other chemicals used were of the purest analytical grades commercially available.

**Blood samples:** Blood samples were collected from confirmed HbSS patients by the personnel of the Hematology unit of the University of Port Harcourt Teaching Hospital (UPTH). Permission for its usage was granted by the Medical and Dental Council of Nigeria.

**The Spices:** The selected indigenous spices *X. aethiopica* and *M. myristica* were purchased from a local market in Owerri metropolis, Imo State of Nigeria and confirmed by a taxonomist of the department of crop science and technology of the Federal University of Technology Owerri, Nigeria. Further identification of the spices was done by Dr. B. C. Ndukwu (a plant taxonomist) of the department of plant science and biotechnology, University of Port harcourt, Nigeria. Specimens of the spices were deposited at the University of Port Harcourt herbarium for reference.

On purchase, the spices (seeds) were collected into sterile glass dessicators and stored in an oven maintained at 70°C until used. The spices (dried samples) were estimated to have been in the market 7 – 8 days before purchase. Two hundred grams (200 g) of each powdered seed of *X. aethiopica* and *M. myristica* were used for the work.

Each of the powdered samples was weighed and two hundred (200 g) gram samples were divided into two equal parts (100 g) or portions. One portion of each of the divided samples was used for crude aqueous extraction process (CAE) and the other subjected to batch-extraction procedures for fat, methanol, butanol and water-soluble fractions (Furnis et al., 1989).

For the crude aqueous extraction, one hundred grams (100 g) of each sample was soaked separately in 200 ml distilled water at 100°C for 12 h. The solution was filtered using Whatman paper No. 1, the filtrate centrifuged at 3000 rpm for 20 min. The supernatant was collected in vials and concentrated at 100°C to get the crude aqueous extracts (CAEs).

The other portions of the samples were separately soaked in 200 ml of chloroform for twenty-four (24) h to defect them and in essence to generate, the fat-soluble (FAS) fraction by filtration. The residue after evaporation to dryness was re-suspended in 200 ml of methanol for 24 h. The filtrates or supernatants were centrifuged, decanted to obtain the methanol/water extract.

Butanol water-partitioning was done with the methanol extract of each of the samples. Exactly, 20 ml of distilled water and 20 ml of butanol were added to each of the methanol extracts after concentrating them. This was left to stand for 24 h and the two-phase liquid separated into the butanol-soluble (BUS) and water-soluble (WAS) fractions respectively. The BUS and WAS fractions were concentrated by rotor evaporation maintained at 80 and 100°C respectively. The volumes were equally recorded. Spectrophotometric readings were taken from UV-spectrophotometer (UNICAM-Spectronic 20 DR; Nordson Engineering Co., Luneburg, Germany).

### Determination of the total free

### Amino acid concentration of the extracts

The ninhydin technique described in the official methods of analysis of Association of Official Analytical Chemists (AOAC, 1984) was used.

The free amino acid concentrations of the extracts were determined with Ninhydrin reagent using phenylalanine as standard and reading the developed purple colour at 570 nm. 0.1% Ninhydrin in acetone was diluted with distilled water in the ratio 1:4. the WAS and CAE extracts were diluted 1:1 with distilled water; the BUS, 1:1 with methylated spirit and the FAS extract, 1:5 with methanol.

Exactly 20 µl each of the diluted extracts were added to 4 ml portion of the diluted Ninhydrin. The resulting solutions were heated to boiling for five minutes (5 min), cooled and the absorbance read on a spectrophotometer at 570 m using distilled water as blank.

### Determination of the major antisickling

### Amino acid constituents of the extracts

The thin-layer chromatographic technique described in the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC, 1984) was used.

Solutions of standard amino acids (the five-naturally occurring antisickling amino acids-Phe, Arg, Tyr, Asp, and Lys were prepared by dissolving 5 mg of each in 0.3 ml portion of 0.1MHCL. The resultant solutions were spotted on one side of thin layer chromatogra-

phic plate (TLC) of dimensions 20 x10 cm using silica-gel as adsorbent.

Diluted portions of CAE, FAS, BUS and WAS were also spotted on the TLC plate alongside with the amino acid standards. The developing solvent was prepared by mixing 80 ml butanol; 20 ml of acetic acid and 20 ml of distilled water in a ratio of 4:1:1, to give a total volume of 120 ml. Ninhydrin was used to develop the plates. The retention factor ($R_f$) values of the standards were recorded and compared with those of CAE, BUS, FAS and WAS fractions.

**Preparation of the blood samples**

Portions (0.20 ml) of the whole blood samples were used for the $Fe^2/Fe^{3+}$ ratio and the sickling reversion experiment while the remaining portions were collected into citrate- anticoagulant tubes. Erythrocytes were isolated from the blood samples by centrifugation at 10,000 (g) for fifteen seconds (15 s) using the bench centrifuge (Nickel-Electro centrifuge). Following careful siphoning of the plasma with Pasteur pipette, the erythrocytes were by repeated inversion suspended in a volume of isotonic saline (0.9% NaCl) equivalent to the siphoned plasma. The erythrocyte suspension was then frozen at 0°C, and subsequently thawed to produce a hemolysate for the hemoglobin polymerization experiment.

**Sickle cell hemoglobin (HbSS)**

**Polymerization inhibition experiment**

The original method of Noguchi and Schetcher (1978) was used for the HbSS polymerization experiment. HbSS polymerization was assessed by the turbidity of the solution (polymerizing mixture) at 700 nm, using 2% solution of sodium metabisulphite as reductant or deoxygenating agent (Iwu et al., 1988). 4.4 ml of 2% sodium metabisulphite ($Na_2S_20_3$), 0.5ml normal saline (0.9% NaCl) and 0.1ml heamoglobin were pipetted into a cuvette, shaken and absorbance reading taken in a spectrophotometer (Unicam-spectronic 20) at 700 nm, every two minutes for 30 min. This represents the control. Distilled water was used as blank for all assays. For the test assays, 4.4 ml of 2% $Na_2$ $S_2$ $0_3$, 0.5 ml of each extract and 0.1 ml hemoglobin (HbSS) solution were pipetted in the cuvette and readings taken as above. The rates of hemoglobin polymerization for the extracts or fractions were estimated by calculating the tangent of a plot of average change in extinction (or change in optical density (ΔOD) versus time in minutes. The rates were expressed as percentages with respect to the control.

**Determination of the $Fe^{2+}/Fe^{3+}$ ratio**

The $Fe^{2+}/Fe^{3+}$ ratio was determined by the methods of Davidson and Henry (1974), while the oxygen affinity of hemoglobin and methemoglobin were measured at 540 nm and 630 nm respecttively. The approach employs lysing 0.02 ml whole blood in 5.0 ml of distilled water and 0.02 ml normal saline. The absorbance of hemo-globin (Hb) and methemoglobin (mHb) were measured at 540 nm and 630 nm to determine the %Hb and % mHb respectively. This represents the control. In determining the effect of the extract on $Fe^{2+}/Fe^{3+}$ ratio, 0.02 ml of each extract was added to 5.0 ml of dis-tilled water and 0.02 ml of blood added and incubated for 60 min in a test tube.

**Sickle cell reversion experiment**

Sickle cell reversal experiment was done using as described by Ekeke et al. (2000). Freshly collected HbSS blood was diluted in 1:2 ratio with 0.9% normal saline/ phosphate buffer (0.2 M, pH. 7.2) solution and then incubated with 2% freshly prepared sodium metabisulphite in a ratio 1:2 for one hour.

At the end of the time, 1.6 ml of the pre-sickled blood in polystyrene tube was mixed with 0.4 ml of the extract. Cells were counted, taking a sample at an interval with the help of a dropping pipette on a slide and then covered with a cover slip. Finger pressure was used to form a thin layer and the slides cover observed using x 40 objective lens by counting the number of sickled and unsickled cells in different fields on the slide. This procedure was repeated for all the samples. The antisickling rate was estimated by measuring the sickling rate for an hour at 10 min intervals, starting from zero time, which is immediately after stirring.

Percent (%) sickling was calculated as:

$$\frac{No.\ of\ sickled\ cells}{Total\ no\ of\ cells\ counted}\ x\ \frac{100}{1}$$

The rate of sickling reversion was calculated as the slope of the curve at zero time.

$$\frac{Percentage\ fall\ (\%)}{Time\ taken\ (\min s)}$$

The time required to reverse 50% sickled cells was calculated from the curve by extrapolation from the 50% relative sickled cell axis to the time axis.

## RESULTS

The results of the different assays and analyses are shown in tables 1-5 and Figures 1 and 2 respectively. Tables 1 and 2 indicate *that X. aethiopica* has more amino acid content than *Monodora myristica* in all the fractions analyzed.

All the fractions of *X. aethiopica* and *M. myristica* demonstrated pronounced antisickling activity (Table 3). The most pronounced antisickling activity was demonstrated by the CAE fraction of *M. myristica* (90% inhibition of HbSS polymerization) while the least was by the FAS fraction of X. aethiopica (72.08% inhibition of HbSS polymerization). All the fractions of X. aethiopica and M. myristica also demonstrated sickling reversion effects (Table 5). The highest rate of reversion was the WAS fraction of *X. aethiopica.* Moreover, the fractions of both spices also demonstrated significant antioxidant effects via $Fe^{2+}/Fe^{3+}$ reduction (Table 4). The highest anti-oxidant effect was demonstrated by the CAE fraction of *M. myristica.*

## DISCUSSION AND CONCLUSION

The amino acids phenylalanine (Phe), tyrosine (Tyr), Arginine (Arg) glutamic acid (Glu) and asparagine (Asn) have been found to exhibit antisickling properties in terms of HbSS polymerization inhibition and improvement of $Fe^{2+}/Fe^{3+}$ ratio (Nwaogiikpe, 1993; Noguchi and Shedder,

**Table 1.** The total free amino acid concentration of the extracts of the samples.

| Fraction/sample | Amino acid conc. (mg/ml) | Vol. of extract (ml) | Total free amino acid conc. (mg/100g) |
|---|---|---|---|
| FAS (*Monodora myristica)* | 5.10 ± 0.1 | 10.60 | 54.06 |
| FAS (*Xylopia aethiopica)* | 7.20 ± 0.0 | 8.45 | 60.84 |
| *BUS* (*Monodora myristica)* | 7.60 ± 0.1 | 10.20 | 77.52 |
| *BUS* (*Xylopia aethiopica)* | 8.00 ± 0.0 | 15.40 | 123.20 |
| WAS (*Monodora myristica)* | 9.20 ± 0.0 | 50.60 | 465.52 |
| WAS (*Xylopia aethiopica)* | 15,00 ± 0.0 | 31.20 | 468.00 |
| CAE (*Monodora myristica)* | 17.00 ± 0.0 | 60.50 | 1028.50 |
| CAE (*Xylopia aethiopica)* | 20.00 ± 0.0 | 83.20 | 1680.64 |

*Values are means of duplicate results.

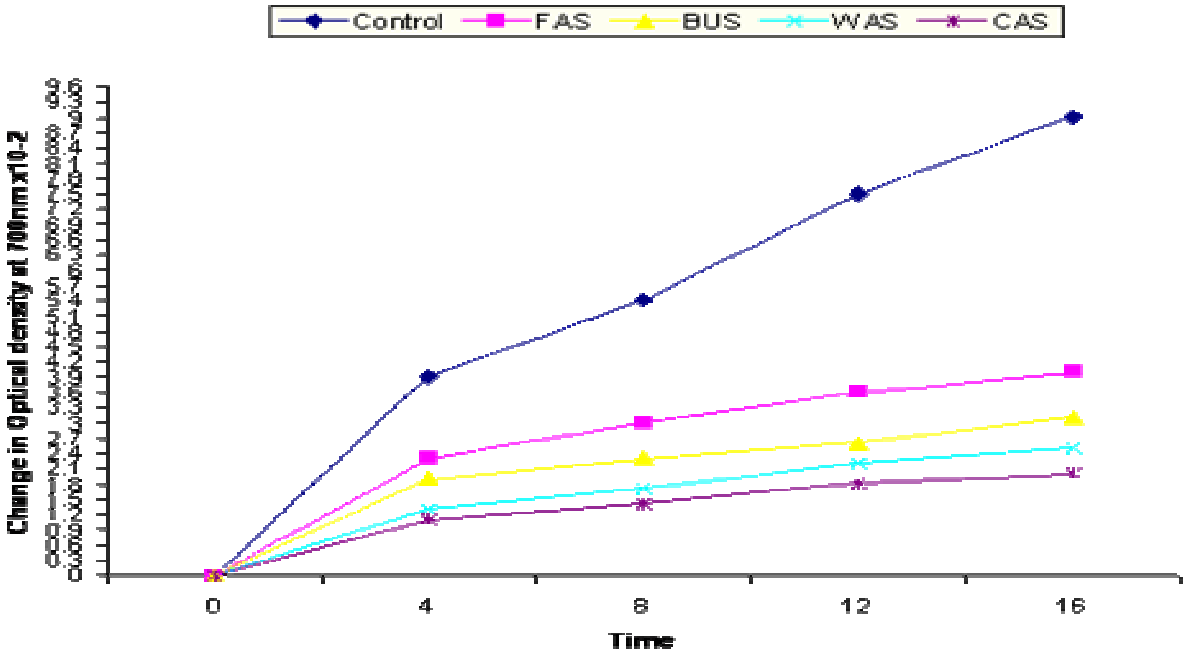


**Figure 1**. The effect of FAS, BUS, WAS and CAE extracts of *Xylopia Aethiopia* on HbSS polymerization.

1978; Ekeke and Shode, 1990; Nwaoguikpe and Uwakwe, 2005).

These amino acids were identified in the various fractions of *M. myristica and X. aethiopica* respectively. The FAS, BUS, WAS and CAE extracts of the samples were able to inhibit sickle cell hemoglobin polymerization remarkably. This antisickling potency can be attributed to the preponderance of those antisickling amino acids already mentioned and other vitamins like vitamin C, E, and sugars already identified in the samples (Nwachukwu, 2000). Vitamin E, amino acids and sugars have been found to inhibit the formation of dense cells of HbSS blood in *vitro* (Ohnishi and Ohinishi, 2001; Ekeke et al., 2001). These results suggest that the extracts must have offered protection to the erythrocytes membranes of HbSS blood from oxidative injury by reactive oxygen species (ROS), thus preventing membrane deformation, haemolysis and the formation of dense cells (Ekeke et al., 2000; Ekeke et al., 2001; Iwu et al., 1988; Noguchi and Schetcher, 1978).

The WAS and CAE fractions of the samples were able to improve the $Fe^{2+}/Fe^{3+}$ ratio, hence facilitating the conversion of methemoglobin to hemoglobin, increasing the oxygen affinity of sickle cell hemoglobin.

From the results, the crude aqueous extracts (CAE) exhibited the highest antisickling effectiveness with respect to the $Fe^{2+}/Fe^{3+}$ ratio, HbSS polymerization inhibition and the reversion of sickled erythrocytes. The advantage of using “non-defatted” aqueous extracts (CAEs) for inhibittion experiment lies in the notion that, for more effective

**Table 2.** The major amino acids identified by TLC in the fractions of the samples.

| Sample | Fractions | Amino Acids Identified |
|---|---|---|
| *Xylopia aethiopica* | FAS | Arg, Phe |
| *Xylopia aethiopica* | BUS | Tyr, Phe |
| *Xylopia aethiopica* | WAS | Tyr, Phe, Arg. |
| *Xylopia aethiopica* | CAE | Phe, Glu, Asp |
| *Monodora myristica* | FAS | Asp, Phe |
| *Monodora myristica* | BUS | Phe, Asp, Arg |
| *Monodora myristica* | WAS | Phe, Asp, Arg, Gly |
| *Monodora myristica* | CAE | Phe, Glu, Arg, Asp, Tyr |

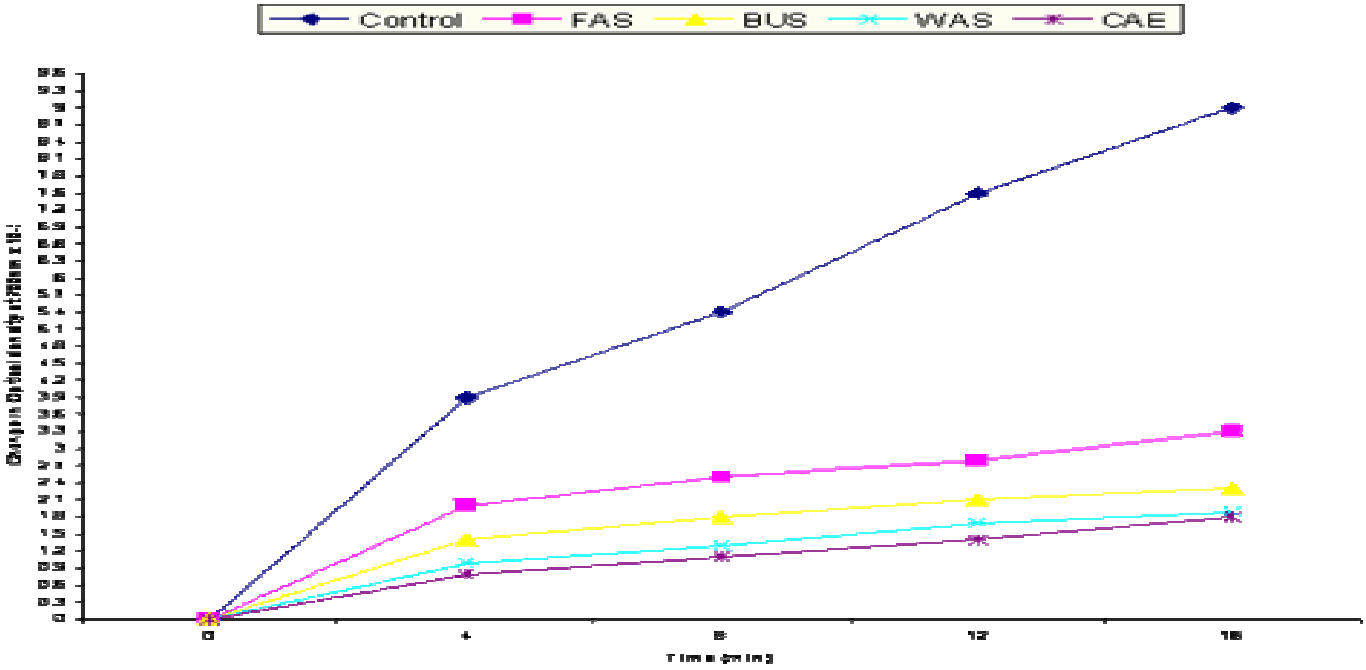


**Figure 2.** The effect of FAS, BUS, WAS and CAE extracts of *Monosora myristica on* sickle cell (HbSS) polymerization

**Table 3.****The rates of polymerization, relative percent polymerization and relative percent inhibition of HbSS by the fractions of the samples at 100 µm PHE equivalence (*In vitro* Assay).

| Sample | Fraction | Rates of polymerization | Relative % polymerization | Relative % Inhibition |
|---|---|---|---|---|
| CONTROL (HbSS) | - | 0.00551± 0.0 | 100.00 | 0.00 |
| *Xylopia aethiopica* | FAS | 0.00154 ±1.0 | 27.95 | 72.08 |
| *Xylopia aethiopica* | BUS | 0.00089±0.0 | 16.15 | 83.85 |
| *Xylopia aethiopica* | WAS | 0.00091±0.0 | 16.52 | 83.48 |
| *Xylopia aethiopica* | CAE | 0.00068±0.1 | 12.34 | 87.66 |
| *Monodora myristica* | FAS | 0.000125±0.1 | 22.69 | 77.31 |
| *Monodora myristica* | BUS | 0.00095±0.1 | 17.24 | 82.76 |
| *Monodora myristica* | WAS | 0.00082±0.0 | 14.88 | 85.12 |
| *Monodora myristica* | CAE | 0.00005±0.1 | 9.07 | 90.93 |

**The results are averages of duplicate assays.

**Table 4.** [+]*In vitro* effect of was and CAE fractions of the samples on the $Fe^{2+}/Fe^{3+}$ ratio of sickle cell blood at a concentration of 40 µm PHE equivalence.

| Sample | Fraction | %Hb | %mHb | $Fe^{2+}/Fe^{3+}$ | % Increase |
|---|---|---|---|---|---|
| HbSS Blood | Control | 93.27+0.1 | 6.73+0.0 | 13.86 | 0.00 |
| *Xylopia aethiopica* | WAS | 94.28+0.1 | 5.72+0.0 | 16.48 | 18.90 |
| *Xylopia aethiopica* | CAE | 94.01+1.0 | 5.99+0.1 | 15.69 | 13.20 |
| *Monodora myristica* | WAS | 96.19+1.1 | 3.81+0.0 | 25.25 | 82.18 |
| *Monodora myristica* | CAE | 96.68+0.0 | 3.32+0.0 | 29.12 | 110.10 |

[+]The results are means from triplicate determinations.

**Table 5.** The effect of was and CAE fractions of the samples on sickle cell erythrocyte reversion at a final Assay Conc. Of 20 µM PHE equivalence.

| Fraction/Sample | Phe equivalent | Initial rate of sickle cell reversion | Time required to revert 50% sickle cells (mins). |
|---|---|---|---|
| *Xylopia aethiopica(WAS)* | 1.20 | 1.40 | 30.0 |
| *Monodora myristica(WAS)* | 1.31 | 1.25 | 28.0 |
| *Xylopia aethiopica(CAE)* | 1.41 | 1.20 | 26.0 |
| *Monodora myristica(CAE)* | 1.46 | 1.33 | 22.0 |

non-toxic therapy, it is the non-defatted portions that contain all the antisickling substances such as amino acids, sugars, proteins, vitamin C, E, Sterols, aminoglycosides, etc.; their concerted antisickling effect would have been responsible for such high potency. Some researchers have equally shown that some chemical substances and their derivatives ingested by lactating/nursing mothers have been found in their breast-milk or mammary glands (Denit, 2004) *X. aethiopica and M. myristica* are extensively used in food preparation for nursing mothers in Southern Nigeria. The extracts of these spices could be used as a target on the prevention of SCD crisis in infants. These indigenous spices/extracts could be used in combination with other foods in the management and prophylactic control of sickle cell crisis and other pathophysiological complications of this and other related syndromes as earlier suggested by Ekeke (1997) and Nwaoguikpe and Uwakwe (2005).

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