Moringa oleifera Lam. (Moringaceae) grown in Nigeria: In vitro antisickling activity on deoxygenated erythrocyte cells

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ABSTRACT

Context: Traditional medicine, which is more available and affordable for the poor uses medicinal plants for the treatment and management of various ailments, including the sickle cell disease (SCD). About 24 million Nigerians are carriers of this sickled cell gene, while approximately 2.4 million are SCD patients. Moringa oleifera Lam. (Moringaceae) possesses high nutritional value and has been used in folklore medicine to treat various ailments related to pain and inflammation. Chemical, pharmacological and pharmacognostical applications of Moringa oleifera have been reported. Objective: This study investigated the antisickling potential of polar and non-polar extracts of the seed, flower and leaf of Moringa oleifera for the first time. Materials and Methods: Using crude methanol extract, aqueous extract, ethyl acetate and butanol, the in vitro antisickling activities of Moringa oleifera fractions, were evaluated using erythrocyte cells deoxygenated with 2% sodium metabisulphite. p-Hydroxybenzoic acid and normal saline were employed as positive and negative controls. Results: Phytochemical screening revealed the presence of saponins, free anthraquinones, and alkaloids. Extracts of the seed and flower demonstrated a higher \( P<0.05 \) antisickling activity in comparison to the leaf extract. The leaf extract, as well as those of the seed and flower, equally demonstrated a \( P<0.05 \) reversal of sickled erythrocytes. Discussions and Conclusions: These findings suggest that Moringa oleifera may play a role in the management of SCD, by incorporation of its fractions into recipes. More extensive biological evaluations and further studies will be necessary for the chemical characterization of the antisickling principles.

KEY WORDS: Moringa oleifera, non-polar fractions, phytochemicals, polar extracts, sickle cell disease

The diverse range of medicinal uses for Moringa oleifera, include its use as an antioxidant,[1] anticarcinogenic,[9] anti-inflammatory, antispasmodic, diuretic,[3] antiulcer, antibacterial, antifungal[6] and its antinociceptive[7] properties, as well as its wound healing ability has been demonstrated.[8] Additionally, the root bark has been used as an analgesic, axeteric, antihelmintthic, and treatment for heart complaints, as well as for eye diseases, inflammation and dyspepsia.[9,10]

Elujoba et al.[11] reported that the use of traditional medicine in the treatment and management of an array of diseases in the African continent is likely to continue due to Africa’s socio-cultural, socioeconomic heritage, lack of basic healthcare, and support for the rural population. Sickle cell disease (SCD) has become a challenge in the African continent with about 89% of global sufferers of SCD. About twenty-five (25%) of the SCD patients in the world are in Nigeria.[12] Hence, this confers on Nigeria the highest population of sickle cell disease patients in the world. Clinical manifestations of SCD are diverse and fall into three major categories, namely,
Materials and Methods

Plant collection and preparation

The leaves, seed and flowers of *Moringa oleifera* were collected between January and March, 2009 from Ibadan, Oyo state and Sagamu, Ogun State, Nigeria. The plant was identified by a plant taxonomist, Mr. Felix, at the Forestry Research Institute of Nigeria in Ibadan, where a voucher specimen was deposited. The studied plant parts were air dried at room temperature (28±2°C) and was powdered using mortar and pestle. The powdered samples were then stored in airtight containers and properly labeled for further analyses.

Phytochemical tests

Powdered samples for each of the plant parts were used to test for alkaloids, saponins, and tannins. Phytochemical analyses were carried out using standard procedures.[15,16]

Extraction

Each of the dried powdered material (500 g) was macerated with methanol (2 l) for 7 days in large amber bottles and filtered. The filtrate was concentrated using a rotatory evaporator, under reduced pressure. For the aqueous extract, dried powdered materials were macerated in distilled water for 3 days, using the same proportion (500 g) used for the methanol extraction. The filtrates obtained were then partitioned successively with ethylacetate and butanol. The aqueous, ethylacetate, and butanol fractions were concentrated using the rotatory evaporator. Thereafter, each of the different fractions was serially diluted with normal saline (0.9% NaCl), to give 10 and 20 mg/ml which were used for the antisickling assay.

Solvents and chemicals

All the solvents (namely methanol, ethanol, butanol, chloroform, and ethyl acetate) and chemical reagents used in this study were of analytical grade and were procured from SIGMA Chemicals Co. Dorset, UK; and BDH Chemicals Limited Poole, England, respectively.

Blood collection and preparation

Blood (5 ml) was obtained in duplicate from a SCD volunteer by venipuncture after informed consent was given in accordance with approved University protocols. The volunteer, who was in steady state, was a confirmed sickle cell disease patient (HbSS) attending the Haematology Day Care Unit of the Olabisi Onabanjo University Teaching Hospital, Sagamu, Nigeria. Blood was collected in sodium EDTA bottles and the content thoroughly mixed by gentle rolling of the bottle. The blood sample was centrifuged to remove serum, leaving the packed erythrocytes, which had been washed with normal saline, as described by Egwunyomi *et al.*[17]

Bioassay of plant extracts for antisickling activity

Bioassay of both crude methanol extract and the aqueous fraction of the three plant materials for antisickling activity were carried out using two approaches. These were the inhibition of sickling (antisickling) approach and the reversal of sickled erythrocytes. Antisickling activity of the extracts/fractions was evaluated using a modified method.[18] The washed erythrocytes (0.5 ml) were mixed with 0.5 ml of each concentration of the test extracts/fractions, in uncovered test tubes.

Samples were then taken from the different mixtures and the remainder, incubated at 37°C, for 3 h while shaking occasionally. Five drops of sodium metabisulphite (2%) were added to the mixture and this was mixed thoroughly and sealed with liquid paraffin. Duplicate samples were taken from the different mixtures at 0 min, followed by the incubation of the systems at 37°C. Additional samples were taken at 30 min intervals, until four further readings were recorded. Smear preparations and counting of sickled and unsickled cells were achieved by following the method, described by Egwunyomi *et al.*[17] Two types of controls were employed in this bioassay. *p*-Hydroxybenzoic acid (5 mg/ml) was used as a positive control, while normal saline was used as a negative control. The percentage inhibition of sickling was
calculated using the formula given in Moody et al.,[19] while the sickling reversal activity of the different plant extracts/fractions was evaluated by using the procedure described by Oduola et al.[20]

The washed erythrocytes (0.5 ml) were again mixed with 0.5 ml of freshly prepared sodium metabisulphite (2%) in a clean test tube and incubated at 37°C, for 30 min. A drop of this mixture was viewed under the microscope. Equal volumes of normal saline/extract/fraction were added to the blood-metabisulphite mixture in a different test tube and incubated at 37°C, for another 30 min. Samples were taken at 0 min and at 30 min intervals for up to 2 h. The earlier procedure described was again used for smear preparations and the counting of sickled and unsickled cells.[17]

**Statistical analysis**

Data obtained were expressed as means. The statistical significance of differences was assessed using analysis of variance (ANOVA). A two-tailed P value of less than 0.05 was considered to be statistically significant.

**Results**

The yields from the aqueous fraction are seed (6.3%), leaf (17.9%), and flower (42.4%). Extractive yield in methanol, ethyl acetate and butanol for seed are 9.7%, 2.2%, and 3.2% while for leaf are 20.1%, 1.3%, and 0.6%, respectively. It can be inferred that higher yield was observed for the aqueous and methanol fractions. Table 1 shows the results for screened phytochemicals of the investigated plant materials. Saponins were detected in all the three plant organs studied. Free anthraquinone was found only in the flower and alkaloids only, in the seed. The results of the antisickling assay are presented in Tables 2–4. p-Hydroxybenzoic acid being the positive control and as expected exhibited antisickling activity. Other tested extracts/fractions also exhibited antisickling activity. Although the antisickling activities of all the tested extracts/fractions compared favorably with that exhibited by PHBA, the exhibited antisickling activities were found to be concentration dependent.

Results of antisickling bioassay for both AqE and ME of MO seed showed that there was no significant difference (P>0.05) between the antisickling activity exhibited at 10 and 20 mg/ml concentrations, at the end of the 2 h incubation period. However, it is noteworthy that at the lower concentration, the antisickling activity gradually increased before it peaked towards the end of incubation.

Mpiana et al. worked at a concentration range of between 0 and 10 mg/ml for the antisickling activity of anthocyanins from Ocimum basilicum.[21] Antisickling activity of the seeds’ butanol fraction was significantly (P<0.05) higher at 20 mg/ml concentration and that of ethylacetate fraction, at 10 mg/ml concentration. The EA fraction of MO leaf (at both tested concentrations), as well as the BU fraction (at 20 mg/ml), caused lysis of the blood.

Extracts of the seed and flower fractions, demonstrated a significantly higher (P<0.05) antisickling activity than the leaf extract. The aqueous fraction equally exhibited a significantly higher (P<0.05) antisickling activity as the crude methanol extract, also as shown. The seeds’ aqueous extracts exhibited a higher percentage reversal of sickling of all the tested parts. However, sickling reversal was more pronounced at the highest tested concentration (20 mg/ml). Nonetheless, this is in contrast with the pattern of sickling reversal exhibited by the flowers’ aqueous extract, which at a lower concentration of 10 mg/ml, gradually increased until it peaked after an incubation period of 2 h. Contrarily, at 20 mg/ml after an initial increase within 30 min incubation, the percentage sickling reversal decreased until the end of 120 min of incubation. Both butanol and ethylacetate fractions of MO seed and leaf had insignificant (P>0.05) abilities to reverse sickled erythrocytes.

**Discussion**

The contribution of phytochemicals to the antisickling activity of any medicinal plant used in the management of SCD is not in doubt, as many reports have attributed the antisickling properties of such plants to their innate phytochemicals. For instance, *zanthoxylol*, a butyric acid derivative and 1-hydroxybenzoic acid, which were isolated from Fagara zanthoxyloides Lam. (Rutaceae) have been suggested to be responsible for the antisickling activity of this plant.[18] *Moringa oleifera* has been reported to contain a rich store of elements like zinc, which possesses antisickling activity, as well as organic acids. Drumstick leaves are also rich sources of flavonols, such as, kaempferol and 3'-OMe quercetin. A flavone, acacetin, and a glycoflavone 4-OMe Vitexin were also detected. Phenolic acids that have been identified, include melilotic acid, p-coumaric acid, and vanillic acid,[22] which could be responsible for the exhibited antisickling activities.

Phytochemical examination of the herbal formula extract, Ajaworon (used for SCD management in Nigeria) whose main constituent is the root of *Cissus populnea* Guill and Perr. (Vitaceae) was found to contain anthraquinones, steroidal and cardiac glycosides, while alkaloids and tannins were absent.[19] This study consequently lends credence to the earlier affirmed position considering the phytochemical compositions of the investigated plant parts vis a viz their corresponding exhibited antisickling activity.

Both the seed and flower fractions of *Moringa oleifera* exhibited a significantly higher antisickling activity, when compared with the leaf extract. This could be attributed to the fact that the latter had only saponins, while the former duo had anthraquinones and alkaloids in addition to saponins. The fact that both butanol and ethylacetate fractions of MO leaf could cause lysis of erythrocytes depending on the concentration used, is an important factor

| Plant part | Combined anthraquinone | Free anthraquinone | Alkaloids | Saponins | Tannins |
|------------|-----------------------|--------------------|-----------|----------|--------|
| Leaf       | -                     | -                  | -         | +        | -      |
| Flower     | -                     | +                  | -         | +        | -      |
| Seed       | -                     | -                  | +         | +        | -      |
that will caution against the inclusion of MO leaf in the recipes for SCD treatment. It has been reported that the mode of preparation of traditional recipes, as stipulated by the herb seller, was by decoction with clean water.[17] The observed significantly higher \( P < 0.05 \) antisickling activity of aqueous extract in this study, supports this, and it is believed that oxidative damage to cells is responsible for the activation of KCl-co-transport in sickled erythrocytes.[23] The sickled cell erythrocytes being fragile and dehydrated require that minerals and antioxidants be constantly supplied to maintain hydration of the cells and membrane integrity.

Consequently, the contribution of micronutrients and the antioxidative properties of some plants, to their antisickling properties have been investigated. Such plants include, aged garlic,[24] M. charantia L. (Cucurbitaceae).[25] and Cymbrogon citratus Stapf. (Gramineae).[26] Incidentally, the antioxidative properties of Moringa oleifera had been reported in literature.[27,28] It then becomes probable that the observed antisickling properties of Moringa oleifera seed and flower fractions in this study could possibly be due to its innate antioxidants and phytochemicals.

In this study, the crude methanol extract of Moringa leaf had an insignificant \( (P > 0.05) \) antisickling activity. Paradoxically, its activity in reversing the sickled cell was highly significant \( (P < 0.05) \). Four cations (\( K^+, Na^+, Ca^{2+}, \) and \( Mg^{2+} \)) have reportedly come into prominence in modulating the ionic pathways involved in the dehydration process.[29] Additionally, it was reported that the ensuing electrolyte imbalances are triggered by diffusional and osmolytical activities. Previous reports have evaluated the mineral contents of Moringa oleifera leaves.[30,31] On understanding that cations such as \( K^+, Na^+, Ca^{2+}, \) and \( Mg^{2+} \) (which are implicated in the process of sickling) may be important parameters in sickle cell management,[32] the involvement of some of these cationic contents of Moringa leaf in the modulation of the ionic pathways of the sickled SS erythrocytes, being responsible for the reversal of sickling observed in the present study is plausible. However, this should be further investigated.

Fahey[1] in his study asserted that Moringa oleifera is native to the Sub-Himalaya tracts of India, Pakistan, Bangladesh and Afghanistan but the fact that Nigeria carries 25% of the 89% SCD sufferers in Africa,[12] could be the reason that the plant is being lately propagated in Nigeria. None of the traditional recipes that are used in SCD management in Nigeria contained Moringa oleifera as a constituent to the best of our knowledge. Therefore, this study which is the first to test the antisickling

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### Table 2: Antisickling effect (% inhibition of sickling) of methanol, aqueous, butanol, and ethylacetate fractions of Moringa oleifera seed

| Time of incubation (min) | Normal saline | \( p \)-Hydroxy benzoic acid (PHBA) | Methanol extract* Concentration (mg/ml) | Aqueous fraction* Concentration (mg/ml) | Butanol fraction** Concentration (mg/ml) | Ethylacetate fraction** Concentration (mg/ml) |
|---------------------------|---------------|---------------------------------|----------------------------------------|--------------------------------------|----------------------------------------|----------------------------------------|
|                           |               |                                 | 20.0                                   | 10.0                                 | 20.0                                   | 10.0                                   |
| 0                         | - (0)         | 0 (0)                           | 0 (0)                                  | 0 (0)                                | 0 (0)                                  | 0 (0)                                  |
| 30                        | - (2)         | 75 (70)                         | 75 (61)                                | 58 (43)                              | 75 (65)                                | 33 (35)                                |
| 60                        | - (4)         | 90 (80)                         | 85 (63)                                | 76 (42)                              | 85 (68)                                | 62 (30)                                |
| 90                        | - (5)         | 92 (82)                         | 86 (65)                                | 85 (38)                              | 86 (71)                                | 79 (28)                                |
| 120                       | - (8)         | 92 (82)                         | 92 (67)                                | 87 (36)                              | 93 (72)                                | 91 (26)                                |

\*P > 0.05; \**P < 0.05; values in brackets are percentage sickling reversal of 2% metabisulphite induced sickled cells

### Table 3: Antisickling effect (% inhibition of sickling) of methanol, aqueous, butanol, and ethylacetate fractions of Moringa oleifera leaf

| Time of incubation (min) | Normal saline | \( p \)-Hydroxy benzoic acid (PHBA) | Methanol extract** Concentration (mg/ml) | Aqueous fraction** Concentration (mg/ml) | Butanol fraction** Concentration (mg/ml) | Ethylacetate fraction** Concentration (mg/ml) |
|--------------------------|---------------|---------------------------------|----------------------------------------|--------------------------------------|----------------------------------------|----------------------------------------|
|                          |               |                                 | 20.0                                   | 10.0                                 | 20.0                                   | 10.0                                   |
| 0                        | - (0)         | 0 (0)                           | 0 (0)                                  | 0 (0)                                | 0 (0)                                  | 0 (0)                                  |
| 30                       | - (2)         | 75 (70)                         | 0 (50)                                 | 0 (48)                               | 75 (55)                                | 16 (40)                                |
| 60                       | - (4)         | 90 (80)                         | 4 (53)                                 | 2 (57)                               | 90 (60)                                | 68 (63)                                |
| 90                       | - (5)         | 92 (82)                         | 10 (60)                                | 8 (50)                               | 92 (62)                                | 76 (70)                                |
| 120                      | - (8)         | 92 (82)                         | 20 (64)                                | 14 (47)                              | 92 (62)                                | 79 (80)                                |

\*P < 0.05; values in brackets are percentage sickling reversal of 2% metabisulphite induced sickled cells. L means lysis

### Table 4: Antisickling effect (% inhibition of sickling) of methanol and aqueous fraction of Moringa oleifera flower

| Time of incubation (min) | Normal saline | \( p \)-Hydroxy benzoic acid (PHBA) | Methanol extract** Concentration (mg/ml) | Aqueous fraction** Concentration (mg/ml) |
|--------------------------|---------------|---------------------------------|----------------------------------------|--------------------------------------|
|                          |               |                                 | 20.0                                   | 10.0                                 |
| 0                        | - (0)         | 0 (0)                           | 0 (0)                                  | 0 (0)                                |
| 30                       | - (2)         | 75 (70)                         | 58 (40)                                | 58 (35)                              |
| 60                       | - (4)         | 90 (80)                         | 77 (28)                                | 68 (40)                              |
| 90                       | - (5)         | 92 (82)                         | 82 (23)                                | 76 (48)                              |
| 120                      | - (8)         | 92 (82)                         | 86 (22)                                | 79 (50)                              |

\*P < 0.05; values in brackets are percentage sickling reversal of 2% metabisulphite induced sickled cells.
effects of a common edible plant in Sub-Saharan Africa where sickle cell disease is most prevalent presents a platform to explore the use of *Moringa Oleifera* for the management of sickle cell disease patients.

**Conclusions**

Findings from the present study have indicated for the first time the antisickling potentials of the seed and flower of *Moringa oleifera*. This suggests the plant could be a valuable source of antisickling agents. The fact that *Moringa oleifera* exhibits antiurolithiatic properties may also advance its use in SCD of antisickling agents. The fact that oleifera.

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