International Journal of Biomedical Research
ISSN: 0976-9633 (Online); 2455-0566 (Print)
Journal DOI: https://doi.org/10.7439/ijbr
CODEN: IJBRFA

Original Research Article

In vitro Anti-drepanocytary (anti-sickle cell anemia) and membrane stability potential of mishenland polyherbal extract on Sickle Red Blood Cells

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***Article History:**
Received: 08/10/2018
Revised: 17/10/2018
Accepted: 17/10/2018
DOI: https://doi.org/10.7439/ijbr.v9i11.4928

Abstract

Background: Drepanocytosis (sickle cell anaemia) is a genetic disorder that causes stiff, rod-like sickle-shaped haemoglobin in red blood cells (RBCs) and consequently poses serious health complications. We investigated in vitro anti-sickling potential of a novel polyherbal formula (MPF).

Methods: Sickling of RBCs induced with 2%sodium metabisulphite (MBS) was followed by treatment with MPF mixtures in different saline concentrations (7mg/ml, 9mg/ml, 14mg/ml and 28mg/ml). The red cell morphology was examined microscopically. Percentage sickling was quantified 30-minute interval at 37^0C for 2-hours. Effect of the MPF on membrane stability of RBCs was analyzed using osmotic fragility testing.

Results: Qualitative phytochemical screening demonstrated the presence of some secondary metabolites namely; alkaloids, glycosides, phenols, saponin, tannin and terpenoids. Sickling of RBCs induced by MBS was inhibited by MPF. This antisickling effect was directly proportional to concentration of the MPF, dose-dependently. There was significant difference ($P<0.05$) between MPF-treated and untreated sickle cell counts. Osmotic fragility curves obtained from MPF-treated RBCs showed leftward shift against the untreated control indicative of increased membrane stabilization and/or haemolytic resistance.

Conclusion: MPF demonstrated significant anti-sickling and erythrocyte membrane stability properties. These effects under hypoxia signify a promising effect of the bioactive components as probable drug candidates against sickling of red cells.

Keywords: Drepanocytosis; erythrocyte membrane stability; *in vitro* antisickling; Mishenland polyherbal formula; phytochemical screening; sickle cell anaemia.

1. Introduction

Drepanocytosis, also known as sickle cell anaemia (SCA) is the commonest sickle cell disease (SCD) in Africa with high mortality rate [1, 2]. SCD is a genetic disorder that causes deformed, rigid, rod-like and sickle-shaped red blood cells (RBCs) occasioned by abnormal β-globin gene. This gene defect causes substitution of β-6 glutamic acid for valine thereby leads to severe reduction in haemoglobin solubility in sickle cells when oxygenated [3, 4]. Also, at low oxygen tension, haemoglobin S (HbS) goes through polymerization, resulting in deformation of RBCs, which assumes a typical sickle shape [5].

Sickling of red cells increases the risk of blood flow obstruction resulting in various serious complications such as sickle cell crisis (sudden and severe pain), severe haemolytic anaemia and multiple organ damage involving heart, brain, lungs, kidneys, skin, penis, skeleton, eyes and spleen [6].

SCD affects millions of people worldwide. About 75% of all patients live in sub-Saharan Africa [7]. Annually, Nigeria records the highest incidence of this disorder worldwide, with approximately 91,011 birth defects [8] and about 100, 000 infant deaths [9].

Meanwhile, many drugs that are currently used in the management of SCD today are associated with cytotoxic effects. Most times, these drugs are expensive and often less effective. As a result, it urgently becomes necessary to further investigation in search for better, non-toxic, efficacious and affordably accessible therapies from the available phytochemical agents [10]. Previously, many studies have shown positive outcomes from the use of various phytochemical agents for managing SCDs [2,10,11]. Precisely, tannin from ethanolic extract of root of U. Afzeliit [12], Flavinoids and cardiac glycosides from extracts of every part of U. Chamae [13] have been proved to posses anti-sickling activity under hypoxia condition. Although there are many findings regarding phytomedicinal use to ameliorate SCA so far, yet availability of quality and genuine varieties are very much in demand [14].

In this present study, a combined medicinal plants' product named Mishenland polyherbal formular (MPF) was put into consideration following consultation with local herbalists. Mishenland contains six different medicinal plant species namely: *Uvaria afzelii* (Gbogbonse or Anikan wogba-arun in Yoruba), *Securidaca longipedunculata* (Ipeta in Yoruba), *Sorghum bicolor* (Poroporo Okababa in Yoruba), *Momordica charantia* (Ejinrin in Yoruba), *Phyllanthus amarus* (Eyin Olobe in Yoruba) and *Dialium guineense* (Awin in Yoruba). We investigated anti-sickling and erythrocyte membrane stability effects of the recipe from this novel polyherbal formulation and scientifically examined its validation *ex vivo*.

2. Materials and Methods

2.1 Plant materials

The following plant species with different parts were selected for antisickling preparation: *Uvaria afzelii*-roots, *Securidaca longipedunculat*-root barks, *Sorghum bicolor*-leaves, *Momordica charantia*-leaves and seeds, *Phyllanthus amarus*-leaves and *Dialium guineense*-leaves and barks. The herbs were obtained fresh from different parts of Ogun and Osun States in Nigeria. They were taxonomically identified and authenticated at the Herbarium, Department of Botany, Olabisi Onabanjo University, Ago-Iwoye, Nigeria.

2.2 Preparation of Mishenland polyherbal formula

Mishenland polyherbal formula (MPF) or recipe was prepared combining various parts of respective plant materials previously dried in shed for three weeks. Each dried herb was crushed, coarsely powdered and then finely blended using mortar and pestle and electric blender to obtain a desired consistency following a previous method [15].

Similar to the usual mode of preparation stipulated by the local herbalists, best described as decoction in clean water, 100g each of individual herb powder was then weighed for similar preparation. The herb substances were mixed geometrically using a blender and dissolved in one and half liter (1.5L) of 50% ethanol–water solution in a conical flask. The mixtures were left for 72 hours under intermittent stirring with a clean spatula, following modified methods of [12,16].

The extract was filtered using Whatmann filter paper No. 42 (125 mm) and concentrated to dryness. The percentage yield was 7.3%. A working solution of 28% (w/v) of the plant extract was prepared with distilled water and then stored at 4°C until the time of analyses.

2.3 Ethical approval

A written consent was obtained from the participants prior to their recruitment. Also, the study protocol was approved by the Ethics Committee of Olabisi Onabanjo University, Ago-Iwoye and was duly followed as approved. Ethical clearance on the use of sickle cell (HbSS) blood was strictly observed according to the international rules.

2.4 Phytochemical screening of Mishenland formula

Qualitative analyses of phytochemical constituents of the MPF extract was carried out using modified standard procedures [17, 18] as described in Table 1.

Table 1: Qualitative analysis of phytochemical constituents for Mishenland polyherbal extract

Phytoconstituents	Test	Observation
Alkaloids (Wagner's reagent test)	2ml extract + 5 drops of Wagner's reagent	Reddish brown precipitate
Anthraquinones (Borntrager's Test)	0.5ml extract + 10ml $FeCl_3$ + diluted HCl + 5ml Benzene (shaken) + 5ml NH_3 (10%)	No colour change
Flavonoids	1ml extract +6 drops 10%NaOH+ 2 drops diluted HCl	Initial intense yellow coloure remained
Glycoside (Keller-Killani test)	5ml extract + 1 drop $FeCl_3$+ 2ml CH_3COOH +1ml conc. H_2SO_4	Brown ring at the interface; characteristic of cardenolides
Phenol	4ml extract + 2ml ethanol + 2 drops $FeCl_3$ 5%	Red colour
Phlobatannins (Precipitate Test)	2ml extract + 2ml HCl (1%v/v) + heat	No colour change
Quinones (HCl test)	2ml extract + 3drops conc. HCl	No colour change
Saponins (Foam Test)	(a) 2.5ml extract + 1.5ml H_2O + heat (shaken) (b)Boiled extract + Olive oil (3 drops)	Froth appears Emulsion forms
Steroids (Liebermann-Burchard test)	0.5ml extract + 3dropsl $CHCl_3$ + 2drops $(CH_3CO)_2O$ + 2ml conc. H_2SO_4	No colour change
Tannins (Braymer's Test)	0.5ml extract + 20ml H_2O + 2-3 drops $FeCl_3$ (5%)	Blue-black precipitate
Terpenoids (Salkowski test)	5ml extract + 2ml $(CH_3CO)_2O$ + 3ml conc. H_2SO_4	Deep red coloration

***Note:** Each observation indicates result of three repeated experiments

Table 1 shows phytoconstituents of Mishenland polyherbal extract after qualitative analyses. Various established methods were employed in testing the presence of these secondary metabolites. The results of the investigations have been summarized later under results.

2.5 Preparation of biological material

Fresh blood was collected from antecubital vein of the stable sickle cell patients (participants) without crises at the time of recruitment. The ages ranged between 18 and 25 years, and both sexes were included. Blood samples were preserved in EDTA bottles for the investigations. We further confirmed their haemoglobin phenotype using electrophoresis method following their medical history.

2.6 Isolation of red blood cells

Serum and buffy coats were separated by centrifugation at 5,000 rpm for 10 mins. The red blood cells (RBCs) were washed three times in isotonic phosphate-buffered saline (IPBS) (pH 7.4; 140 mM NaCl, 5 mM KCl and 5 mM glucose in 10mM phosphate buffer conc. using 1:5 v/v) as previously reported [16]. The RBCs were then re-suspended in IPBS solution (1:1 v/v; 50% suspension). The red cell suspensions used in this study were freshly prepared on daily basis.

2.7 Polymerization inhibition (anti-sickling) test

Principle: When sickle red blood cells (SS-RBCs) are deoxygenated by addition of a reducing agent, the RBCs turn sickle and maintain this abnormal shape until when re-exposed to sufficient oxygen. The ability of a drug to prevent or inhibit sickling in this deoxygenated state is then determined by counting the number of sickle cells over a period of time.

Procedure: The suspended RBCs (100 µl) on incubation in IPBS (at 37 °C for ≥30 min. in a water bath) was mixed with graded dilutions / concentrations of the MPF (7mg/ml, 9mg/ml, 14mg/ml and 28mg/ml) in test tubes. Samples were taken from different mixtures at varying time intervals (30, 60, 90 and 120 minutes) incubated at 37°C, while shaking intermittently.

***2.8 In vitro* induction of red cell sickling**

100 µl of 2% sodium metabisulphite (SMB) were added to the mixtures of pre-incubated RBCs in MPF solutions, mixed thoroughly and sealed with liquid paraffin to keep out air (maintaining hypoxia). Samples were then taken from different mixtures, after which the samples were further re-incubated at 37°C. The samples were taken at 30 min. intervals until four readings were recorded on a row. Both the SS-RBCs and total cells (including normal and abnormal) were enumerated from five different fields randomly selected across the slide after counting at least 100 cells altogether. The procedure was repeated five times each for every sample mixture while the average counts were reported. Normal saline (NaCl 0.9%) without the extract was used for positive control.

2.9 Morphological evaluation of red blood cells

After the RBCs were observed, counted and analyzed under an Olympian light microscope. The procedure for smear preparation and counting of sickled and unsickled cells followed a modified method of [12]. The counting was done by a Principal Medical Laboratory Scientist majoring in haemtology. Normal cells were considered for the RBCs that reasonably resembled the shape of a classical biconcave disc and including those with central parlour, while abnormal were considered for the cells (drepanocytes) with elongated, crescent, star-like, rod-like, wrinkle, or bolt-shaped.

The percentage inhibition of sickling was calculated using the formula of [19].

% Sickling = Number of sickle cells X 100 / total cells counted.

2.10 Osmotic Fragility Test

The fragility of RBCs (ability of RBCs to resist haemolysis) was determined by mixing fresh RBCs (1:1 v/v; 50% suspension in phosphate buffer) with graded concentrations of hypotonic saline solutions (pH7.4) at 1:200 blood-hypotonic solution ratio. Two tubes were used each for dilutions labeled as treated and untreated of every blood sample, respectively. Concentrations ranging from 0.2% to 0.8%NaCl were made up in 5 ml pipettes. A separate mixture of standard isotonic solution (0.9% NaCl w/v) was used as control.

Untreated preparation: A 10µl sample of washed sickle RBCs was added to 1990µL (after removing 10µL away from 2000µL) of each graded hypotonic saline solution and immediately mixed by inverting several times. The tubes were allowed to stand for 150 mins at room temperature.

Treated preparation: To determine the effect of the formulation, saline solution containing MPF extract was prepared. Hundred mirolitres (100µl) of MPF (28 and 14 mg/ml) were added to 1900µL of each hypotonic saline solution (by 1:20 v/v) separately. Then, 10µl of RBCs was added (after removing 10µL away from 2000µL of the mixture) following a modified method of [20].

Standard preparation: A 10µl sample of washed SS-RBCs was added to 1990µL of isotonic saline solutions, 0.9% NaCl (by 1:200 v/v) mixed accordingly following the same method for both treated and untreated preparations. The number of nonhaemolysed RBCs in each saline concentration was counted manually using hemocytometer (Neubauer's cell counter) under eyepiece lead microscope (Olympus BX43) using 40X objective power. Haemolysis was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{\text{No of RBCs in 0.9\% NaCl standard - No of RBCs in hypotonic grade}}{\text{No of RBCs in 0.9\%NaCl standard}} \times 100$$

2.11 Statistical Analysis

Results were reported as mean ± standard error of mean (SEM) of five determinations. SPSS V20.0 was used for data analysis. The statistical significance of difference was calculated using Kruskal-Wallis and Mann-Whitney *U* tests. $P \leq 0.05$ considered as significant.

3. Results

Blood samples from sickle cell (SCD) stable patients were obtained to assess the potency of Mishenland polyherbal formula in preventing sickling of red blood cells. The results are presented in tables, micrographs, and chart below.

Qualitative phytochemical screening demonstrated presence of some secondary metabolites, namely; Salkaloids, glycosides, phenols, saponin, tannin and terpenoids. Among those tested negative and probably absence were anthraquinone, flavonoids, phlobotain, quinones and steroids.

Table 2: Time course of the sickling of SS-RBCs in extract-treated preparations by red cell count

Conc. (mg/ml)	% number (average) of sickling cells at 30-min interval			
	30 mins	60 mins	90 mins	120 mins
[a]Control (0.00)	40.40±0.9	73.10±1.1	77.62±1.6	81.71±1.2
[a]MPD (28.0)	8.91±0.3	17.12±1.3	23.23±0.2	24.17±0.7
[a]MPD (14.0)	15.44±1.5	27.28±0.9	32.52±0.3	35.62±0.4
[a]MPD (9.0)	25.59±0.9	40.36±0.7	50.24±0.8	57.89±0.5
[a]MPD (7.0)	35.06±0.9	65.78±1.2	72.84±0.7	75.04±0.9
[b]**P-value**	0.001*	<0.001*	0.002*	0.001*

[a]Values expressed as mean ± standard error of mean (n = 5); [b] Kruskal-Wallis Test; *Significant at P≤0.05; MPD: Mishenland polyherbal drug formula; **SS-RBCs**: Sickle red blood cells

Table 2 shows the mean number (percentage) of sickle cells counted manually under the microscope following treatment with various concentrations of MPF - extract at 30-minute interval. There were significant differences (P< 0.05) in numbers of SS-RBCs count comparing the effect of various dilutions of MPF-extract against the control (normal saline preparation). Red blood cell counts were recorded using microscopy method at 30-minutes (P=0.001), 60minutes (P<0.001), 90 minutes (P= 0.002) and 120 minutes (P= 0.001) respectively (Table 2).

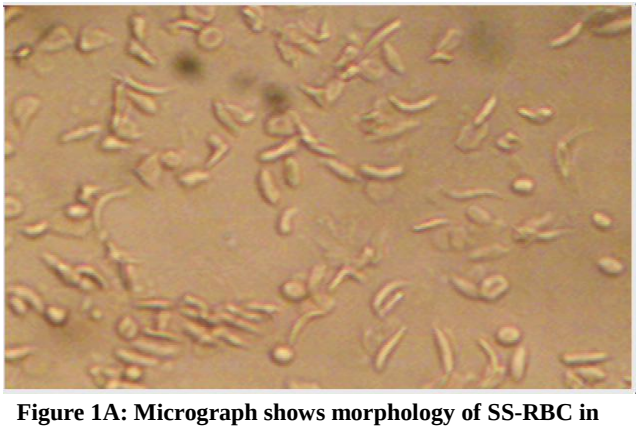

Figure 1A: Micrograph shows morphology of SS-RBC in positive control slide (Mag. X400)

Figure 1A shows photomicrograph (of the control slide) with numerous abnormal shapes of red cells (white arrows) after treatment with a reducing agent (2% $Na_2S_2O_4$) in normal saline at the end of 120 minutes. The blue arrows point toward classical biconcave discs of normal red cells.

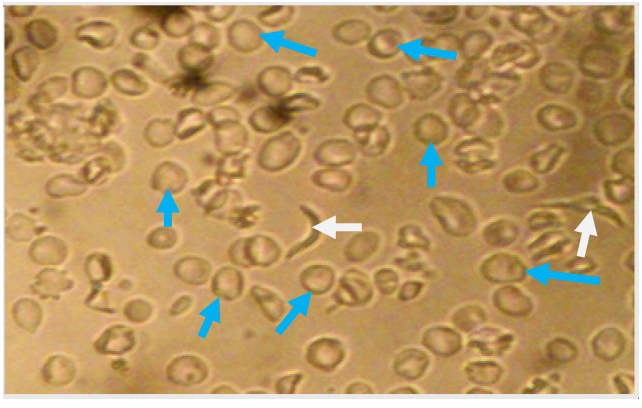

Figure 1B: Photomicrograph shows the effect of MPD-extract on SS-RBC morphology (Mag.X400)

Figure 1B shows photomicrograph where pre-treatment of SS-RBCs with MPD/normal saline dilution inhibited sickling of red cells on exposure to low oxygen tension milieu (after induction with 2% $Na_2S_2O_4$). The micrograph shows more of classical biconcave discs of normal red cells and cells with central parlour (blue arrows) as against the abnormal sickle cells (white arrow).

Table 3: Determination of mean corpuscular fragility indices as effect of MPD-extract on SS-RBCs

	MCF-Index (%)	P-value
MPD-treated cells (28mg/ml)	0.49	0.005[a]
MPD-treated cells (14mg/ml)	0.60	0.008[b]
Untreated cells	0.71	

a: Statistical significant value comparing MPD-treated group (28mg/ml) against the control group; **b**: Statistical significant value comparing MPD-treated group (14mg/ml) against the control group; **MCF**: Mean Corpuscular Fragility; **SS-RBCs**: Sickle red blood cells.

The mean corpuscular fragility (MCF) was determined from the concentration of saline causing 50% haemolysis of the RBCs) [21]. MCF-index was derived through interpolation from osmotic fragility curves of haemolysis (%) *vs.* NaCl concentrations (w/v %), for both treated and untreated groups. Results were presented by graphical representation in figure 2. There were significant differences (P=0.005 and P=0.008) comparing MCF-Index from the plots of MPF-treated (28mg/ml and 14mg/ml) in varying hypotonic saline solutions compared with untreated hypotonic solutions, correspondingly (Table 3).

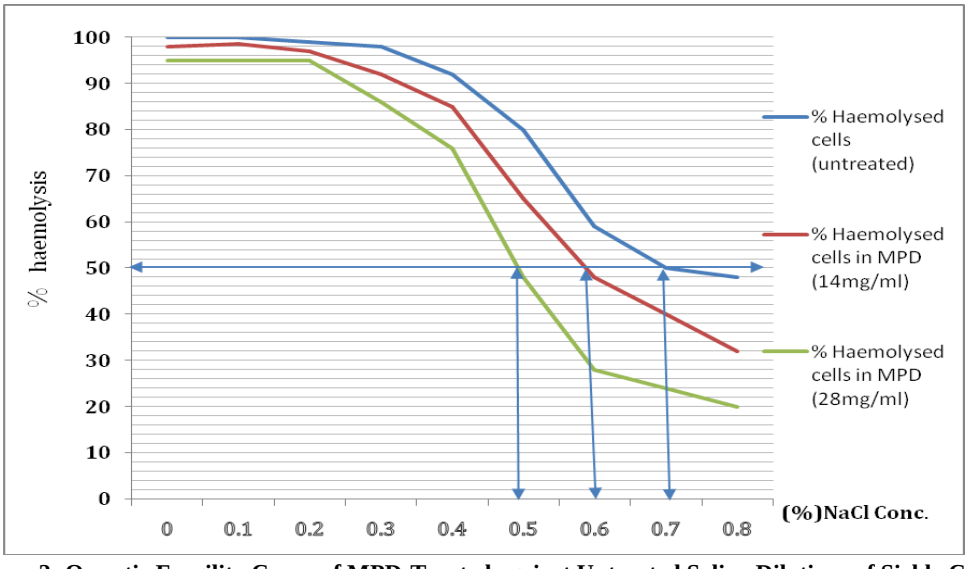

Figure 2: Osmotic Fragility Curve of MPD-Treated against Untreated Saline Dilutions of Sickle Cells

4. Discussion

The use of folklore medicine in the treatment of ailments has been acknowledged since the times immemorial. Recently, plant-derived pharmaceutical substances became of great interest [22]. Various approaches have been tailored toward discovery of drug candidates that can inhibit the polymerization of haemoglobin S (HbS) or increase the oxygen affinity of red cell and thus prevent or reduce occurrence of sickle cell disease (SCD) crises [23].

The present study showed increased number of sickle red blood cells (SS-RBCs) in positive controls left untreated with Mishenland polyherbal extract on exposure to low oxygen tension. In other way, treatment of the same SS-RBCs with extract of Mishenland polyherbal formula (MPF) inhibited the purported sickling by large difference. The treatment with this novel phytomedical product greatly demonstrated reduced number of sickle cells. This anti-sickling effect was also shown to have direct relationship with concentrations of the extract, thus suggesting the potency of MPF to be dose-dependent. Our report is consistent with previous studies [2, 12]. Although, we put different plant species into trial, however, the presence of similar pharmaceutically active secondary metabolites such as alkaloids and phenolics in previous studies [10, 12, 24] indicate possible similar mechanisms of reaction. Besides, in her report, Gbadamosi [25] highlighted various traditional recipes employed for the management of SCD. Majority of these recipes incorporated at least one similar plant species formulated in MPF-recipe of the present study. Pills made of fresh shoot of *P. amarus* denoting mono-plant recipe and a powder of another recipe involving leaves of *S. bicolor* were said to be good anti-sickling phytomedicines.

Accordingly, the outcomes of this present study are also in concordance with previous reports where *U. afzelii* roots [12], *S. longipedunculata* root barks [26], *Sorghum bicolor* leaves, *M. charantia* leaves [11], *P. amarus* leaves [27] and *D. guineense* [28] have been screened for the presence of phytochemicals such as alkaloids, flavonoids, tannins, saponins, glycosides and tannins. In addition, application of phytochemicals such as glycosides, in some plant species for inhibition or prevention of red cell sickling has also been reported [29]. The presence of these active constituents in MPF-extract of the present study may be an indication for anti-sickling property.

Furthermore, effect of MPF on erythrocyte membrane stabilization and their ability to resist haemolysis was further evaluated by comparing the haemolysis rates between the MPF-treated and untreated SS-RBCs using osmotic fragility test (OFT) at varying NaCl concentrations. MPF revealed appreciable erythrocytes membrane protection with reduced mean corpuscular fragility index (MCF-index). That is, osmotic fragility curves showed leftward shift for the two most effective concentrations (14mg/ml and 28mg/ml) against the untreated control and thus indicative of increased RBC membrane stabilization.

When the osmotic fragility of a cell reduces, the osmolytic resistance increases and vice versa [10].

The ability of all these plant extracts to increase resistance to hemolysis as presented in this work is consistent with several earlier reports that showed the efficacy of medicinal plants on the reduction of osmotic fragility of red blood cells [24, 30].

This stabilization effect could be explained by the fact that MPF rendered the SS-RBCs capable of withstanding higher concentrations of NaCl by increasing the volume of the RBCs, reverting the sickling to produce biconcave cells, and thereby, maintaining membrane integrity. This may possibly indicate that MPF effected mechanistically at the cell membrane level and not through the direct interaction with Hb S molecules against the earlier assumption of inhibition of Hb polymerization.

5. Conclusion

Mishenland polyherbal formula (MPF) or extract contains some pharmaceutically important phytochemicals and secondary metabolites. These constituents were able to prevent good percentage number of sickled cells, during induced-hypoxia. The observed anti-sickling effect of MPF, through its active metabolites, controlled the hypoxia-induced sickling *in vitro,* hence may protect sickle cell patients from effects of low oxygen tension, especially from crisis. Mechanistically, MPF-extract showed great potential by possibly inhibiting Hb S polymerization or increase the haemoglobin oxygen affinity indicated by shift in the oxygen dissociation curve leftwards. This effect was dose-dependent under hypoxia, a promising result of its bioactive components as probable drug candidates.

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