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T2MC- A poly-herbal that inhibits polymerization of intracellular sickle hemoglobin and regulates the expression of erythrocyte Ca^{2+} activated K^+ channel

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ABSTRACT

Background & Aim: *Telfairia occidentalis*, *Moringa oleifera* and *Cnidoscolus aconitifolius* are nutritious vegetables used individually, or in different combinations as blood boosters in managing sickle cell anemia in South-Western Nigeria. In this study, we evaluated the antisickling properties of the polyherbal combination, as well as the mode(s) of action, using molecular biology techniques.

Experimental: *T. occidentalis*, *M. oleifera* and *C. aconitifolius* leaves were extracted separately in ethanol after identification by a taxonomist. The extracts were combined in different ratios (TMC, T2MC, TM2C, TMC2) and evaluated at 4 mg/mL concentration for antisickling properties. Ciklavit®, an antisickling nutraceutical, was utilized as the positive control. We evaluated the combination (T2MC) for the rate of inhibition of polymerization of sickle hemoglobin (HbS), erythrocyte membrane stabilization, and rehydration of dense sickle red cells *in vitro*. The regulation of the Gardos channel, erythropoietin (EPO), mitogenesis, and antioxidant genes was assessed *in vivo* by harvesting and analyzing the bone marrows, kidneys, livers of Wistar rats administered with T2MC orally at 50 and 100 mg/kg body weight for 28 days. Folic acid was employed as the positive control.

Results: The T2MC treated samples showed significantly higher ($p<0.05$) antisickling activities (95.4 ± 0.15% inhibition and 95.6 ± 0.28% reversal), and cell membrane stabilization (76.8 ± 0.05%) than the positive controls. Additionally, T2MC reduced the density of sickle red cells by 18.2 ± 0.86%, and showed inhibition of polymerization of HBS comparable to positive controls. Finally, T2MC down-regulated the expression of the Gardos-channel and the erythropoietin genes suggesting that T2MC works by inhibiting the activation of Ca^{2+}activated K^+ channel, thereby preventing cell dehydration.

Recommended applications/industries: T2MC is a potent antisickling nutraceutical and could be used in the management of sickle cell disease.

1. Introduction

Sickle cell disease (SCD) is the commonest hemoglobinopathy amongst the people living in sub-Saharan Africa, Mediterranean regions, Middle East and South-East Asia (WHO, 2001). The condition is most predominant in the tropical districts, however with population migration the disease has spread to most nations of the world (WHO, 2017). In countries such as Nigeria, Republic of Congo, Ghana, Cameroon

and Gabon the incidence is 20%-30% (WHO, 2017). SCD is caused by an A-to-T point mutation resulting in the replacement of the hydrophilic glutamic acid with the hydrophobic valine at the sixth position of the β-globin gene that produces abnormal sickle hemoglobin (Hb S) (Hartwell *et al.*, 2000). As such, in the deoxygenated state, deoxy-Hb S molecules polymerize into elongated fibers that deform the red cells into characteristic "sickled" shapes, causing increase in cell rigidity, decreased cell deformability, and diminished sickle red blood cell (RBC) membrane functionality resulting in dehydration of red cells through K^+ loss with accompanying Cl^- and water movement and a resultant gain of Na^+ (Brugnara *et al.*, 1996). There is no widely available cure for SCD, but some children and adults with the disease have been successfully treated with hematopoietic stem cell transplantation (Dedeken *et al.*, 2014; Hulbert and Shenoy, 2018). In the meantime, there is need to manage the downstream effects of the disease with the use of disease modifiers such as hydroxycarbamide (hydroxyurea) or alternative remedies. Recently, the US Food and Drug Administration (FDA) approved three new therapies: Oxbryta, L-Glutamine, and Crizanlizumab. However, concerns remain about availability and affordability of these novel drugs to the global population of SCD, especially in the developing world, necessitating continued investigations into alternative herbal therapies. Plant derived recipes have been used to treat SCD, but only few of such recipes have been validated scientifically (Egunyomi *et al.*, 2009). Herbal medicines are employed extensively in treating ailments, and are frequently used in combinations compounded from plant sources of different species, growing conditions, and varying biologically active constituents. The mixture of these bioactive ingredients found in many herbal remedies are similar to modern combination therapies. Every component of the traditional recipe used in SCD management has a peculiar role that it is performing. This supports the opinion of Gillete *et al.* (2004) that all constituents of a therapeutic mixture of plants are necessary. Nicosan (an herbal based drug for SCD management) was developed from the aqueous extracts of four plants: *P. guinensis*, *P. osun*, *E. caryophylla* and *S. Bicolor* and was reported to inhibit sickling, delay polymerization of deoxy HbS and directly interacted with the hemoglobin (Iyamu *et al.*, 2002). The combination in this study consists of three nutritious vegetables cooked as soups and used ethno-medically in the management SCD in Southwestern Nigeria. They are *Telfairia occidentalis* Hook, F. (Family Cucurbitaceae), *Moringa oleifera* Lam. (Family Moringaceae) and *Cnidoscolus aconitifolius* (Mill.) I.M. Johnst. (Family Euphorbiaceae). They are used locally by individuals with SCD to manage painful crisis, improve and boost the blood because anemia is one of the features associated with SCD. *Telfairia occidentalis* (TO) is used locally as a blood booster and has been reported to have positive erythropoietic activity (Alada, 2000; Salman *et al.*, 2008; Eseyin *et al.*, 2014). *Cnidoscolus aconitifolius* (CA) is a common vegetable well known for its hematinic effects and its use in managing anemia in patients with SCD (Cyril-Olutayo and Agbedahunsi, 2015; Moura *et al.*, 2019) while *Moringa oleifera* (MO) is used ethno-medically to treat anemia and pains associated with SCD (Cyril-Olutayo *et al.*, 2018). The antisickling effects of each of the three plants have been reported by various researchers (Adejumo, 2012; Atabo *et al.*, 2016; Saturnin *et al.*, 2018; Moura *et al.*, 2019; Cyril-Olutayo *et al.*, 2019), but the antisickling property of the recipe consisting the three plants has not been evaluated. The aims of this study are therefore to authenticate the ethno-medicinal claim, determine the combination of TO, CA and MO with the best antisickling properties and determine the mode(s) of action. This is with a view to standardize the recipe and formulate for use as an herbal product in managing SCD.

2. Materials and Methods

2.1. Preparation of extracts

Telfairia occidentalis Hook. F. (TO), *Moringa oleifera* Lam. (MO) and *Cnidoscolus aconitifolius* Mill. I.M. Johnst (CA) were collected at Obafemi Awolowo University Ile-Ife, Osun State, Nigeria campus with GPS coordinates of 7° 31' 14.7612" N and 4° 31' 49.1340" E. The plants were identified and authenticated by B.E Omomoh and G. Ibhanesebor at the IFE Herbarium, Obafemi Awolowo University, Ile-Ife and voucher specimens deposited with voucher numbers IFE 17254, IFE 17255, and IFE 17256 assigned, respectively. The leaves of each plant were extracted separately by maceration in absolute ethanol for 72 h and dried by using a rotary evaporator. The

ethanol extracts were weighed separately and mixed in different ratios viz: 1:1:1 TO:MO:CA (TMC); 2:1:1 TO:MO:CA (T2MC); 1:2:1 TO:MO:CA (TM2C) and 1:1:2 TO:MO:CA (TMC2), to determine the combination with the best antisickling property.

2.2. Blood collection

Left over blood samples collected from patients with SCD(ages 18-30 years), that attend routine Hematology adult clinic at Obafemi Awolowo University Teaching Hospitals Complex, Ile Ife, (OAUTHC) were used within the first 48 h of collection. Consent was obtained and Ethical approval with certificate no.: IRB/IEC/0004553 from the Ethical Committee of the OAUTHC, Ile-Ife, was obtained for this study.

2.3. Antisickling assay procedures

2.3.1. Inhibitory and reversal model

Whole blood sample was centrifuged for seven mins at 2500 rpm (Centrifuge: Denley) and the resultant packed Hb SS red blood cells (RBC) washed thrice using phosphate buffered saline (PBS, pH 7.0). In the inhibitory model, 0.2 ml of each polyherbal combination (TMC, T2MC, TM2C, TMC2) was incubated with 5 ml of 10% v/v RBC suspension in triplicate test tubes for 1h at 37^{o}C, covered with parafilm. Ultra-pure nitrogen gas was bubbled in for 1h to induce hypoxia. In the reversal model, however, nitrogen gas was first bubbled into 5 ml of 10% v/v Hb SS RBC suspension at 37^{o}C for 1h, covered with parafilm, to induce deoxygenation. After that, 2 ml of each polyherbal combination was added under parafilm, carefully shaken and re-incubated for another 1 h. With the Pasteur pipette, 200 µl of the solution was taken and fixed in 5% v/v buffered formalin solution ready for counting (Cyril-Olutayo and Agbedahunsi, 2015).

2.3.2. Sickle cell hemoglobin polymerization inhibition assay

The polymerization assay was performed according to Iwu *et al*. (1988). 0.5 ml of 0.9% sodium chloride was substituted for the extract as negative control while para hydroxybenzoic acid (PHBA) was used as the reference compound. Test was carried out in triplicates and mean values used for the determination of the rate of polymerization and hence relative inhibition. The rates of change of absorbance at 700 nm over time in minutes, was taken as the index of rate of polymerization determined.

2.4. Red cell fractionation assay

Evaluation of the effect of T2MC on the density of the sickle RBC sub-population was carried out using the *in vitro* red cell fractionation assay (Mackie *et al*., 1987; Akinola *et al*., 1992). Ciklavit® and PBS were employed as positive and negative controls, respectively.

2.5. Membrane stabilizing assay

The assay was carried out according to Sadique *et al*. (1989). T2MC was tested at different concentrations (300, 250, 200, 150, 100, and 50 µg/ml).

The membrane stabilization, expressed in percentage, was estimated thus:

[100 - (absorbance of test drug – absorbance of drug control)/ Absorbance of blood control] × 100

2.6. In-vivo studies

2.6.1. Animal handling

Laboratory animals (male and female Wistar rats) obtained from the animal house of the Adekunle Ajasin University, Akungba-Akoko, were randomly divided into four groups of five rats each. Groups A and B received T2MC at 50 mg/kg and 100 mg/kg body weight respectively while group C represented the positive control group and were administered with folic acid 50 mg/kg. The last group D is the placebo group that received only distilled water. The samples were administered for 14 days. At the end of the study, the rats were fasted overnight and anaesthetized with chloroform and blood samples collected for hematological analysis while tissues of the bone marrow, liver, and kidney were collected for RNA isolation (Osuntokun *et al*., 2017). All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Institutional Animal Ethical Committee (IAEC).

2.6.2. RNA isolation and cDNA preparation

The tissues (bone marrow of tissues femoral shaft, liver and kidney) for RNA isolation were homogenized in 100 µl RNAse cocktail (4 M guanidinium isothiocyanate, 20 mM sodium acetate, 0.1 mM dithiothreitol, 0.5% N-luroylsarcosine, pH 5.5), 200 µl phenol: chloroform: isopropanol (125:49:1 v/v/v)

solution was added to the homogenate and incubated at 4°C for 20 min and separated at 10,000g, (20 min). RNA was pelleted (10,000g, 20 min) from the supernatant (100 µl) after incubating at 4°C for 1 h in ammonium acetate (5 µl, 0.2 M) and isopropanol (100 µl). RNA samples were washed twice in 75% ethanol and dissolved in 25 µl nuclease-free water. The purity (O.D 260/180 > 1.8) and concentration of the RNA were determined spectrophotometrically. DNA contaminant was removed by DNAse I treatment (ThermoFisher Scientific) according to the manufacturer's protocol. 100 ng DNA-free RNA was converted to cDNA straightway using M-MLV reverse transcriptase (GibcoBRL, Carlsbad, CA) in a 20 µl reaction buffer containing 3 µl of random primers, and 1 mM dNTPs, following incubation at 42°C for 52 min. The reaction was terminated at 65°C (5 min) (Osuntokun *et al.*, 2017).

2.6.3. PCR and Gel electrophoresis

PCR was carried out in a final reaction volume of 50 µl containing 25 µl mater mix (Thermo Scientific), 2 µl of cDNA, 1 µl (10 mM) each of forward and reverse primers and 21 µl nuclease-free water (Table 1). The PCR was programed as follows: 95^0C/5 min, 30 cycles of (95^0C/ 30 sec, (Tm-4)^{0}C/45 sec, 72^0C/1 min) and 72^0C for 5 min.

Table 1. Primer set for in vivo molecular study.

Primer name	5′-Forward Sequence -3′	5′-Reverse Sequence -3′
Erythropoietin	AGGCGCGGAGATGGGGGTGC	ATCGGATGTGGGTGGTCATAGG
Erythropoietin receptor	TTGAGAGCAAAGCGGCCCTGCT	ATCGGATGTGGGTGGTCATAGG
rKCCN4	CACCTTGCCCTGGAGAAGA	GTCCTGAGTAGAATGTAGATCGA
rKCC1	CCTGGAGTTGGGTTGTCTAAGA	CATCAGCCCTCACCAGTCATCTC
Ca^{2+}-activated K^+-channel (Kcnma1)	TTTGCTCTCAGCATTGGTGC	TGCAATAAACCGCAAGCCAAA
G6pDD	AGCCTGGCGTATCTTCACAC	GCTCAGAGCTTGTGAGGGTT
Catalase	GTGCATGCATGACAACCAGG	GAATGTCCGCACCTGAGTGA
Glutathione peroxidase (GPX1)	CGGACATCAGGAGAATGGCAA	TAAAGAGCGGGTGAGCCTTC
Glutathione reductase (GSR)	GTATCACGCTGTGACCACGA	TGGATGCCAACCACCTTCTC
p21	GACATCTCAGGGCCGAAAAC	CGGCGCTTGGAGTGATAGAA
p27	CCTTCGACGCCAGACGTAAA	AGCAGTGATGTATCTAATAAACAAGGAATT
β-actin	GTCGAGTCCGCGTCCAC	AAACATGATCTGGGTCATCTTTTCAC

(All oligonucleotides are products of Inqaba Biotec (South Africa). rKCCN4= Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4, rKCCN1= Potassium *intermediate/small conductance calcium-activated channel, subfamily N, member 1, G6pDD= Glucose-6-Phosphate Dehydrogenase).*

2.7. Statistical analysis

Data were expressed as mean ± (SEM), comparisons were made by one-way (ANOVA) followed by Turkey's test using GraphPadPrism 8, and $p \leq 0.05$ was considered significant.

3. Results and discussion

3.1. Antisickling effects of the various combinations

The three plant extracts (TO, MO, CA) were mixed in different ratios to give TMC, T2MC, TM2C and TMC2, and the combination with the highest antisickling activity was determined. All produced significantly ($p<0.05$) higher and better inhibitory activities than Ciklavit®, the positive control but T2MC gave the best antisickling activity of 95.4 ± 0.15 % inhibition and 95.6 ± 0.28 % reversal (Figure 1).

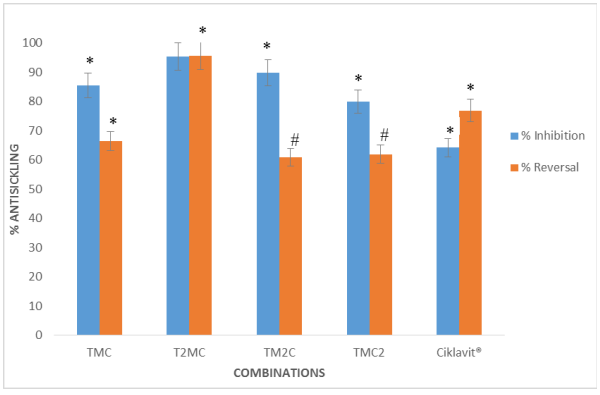

Fig 1. The antisickling results of the various combination mixtures of TO, MO and CA.

KEY: TMC = TO: MO: CA (1:1:1); T2MC = TO: MO: CA (2:1:1); TM2C = TO: MO: CA (1:2:1) TMC2 = TO: MO: CA (1:1:2). **(*)** - mean difference is significant ($p < 0.05$); (#) -not statistically different ($p >0.05$).

Photomicrographs showing Hb AA blood cells, blood film from Hb SS individual in steady state, deoxygenated Hb SS blood cells, the inhibitory and reversal effects of T2MC on Hb S red blood cells are shown in Figures 2-4, respectively. The constituents of each plant in the combination work synergistically contribute to the antisickling property of T2MC.

The rate of decrease in polymerization was observed to increase with time and concentration. The rate of decrease in polymerization at 4 mg/ml is significantly higher than 2 mg/ml > 1 mg/ml > 0.5 mg/ml > 0.25 mg/ml. Interestingly, from 20-30 minutes at 2 mg/ml, there was 100% inhibition of polymerization, which was also observed from 10 to30 minutes at 4 mg/ml (Table 2).

Table 2. Rate of decrease in polymerization of T2MC and PHBA (positive control).

Time (mins)	0.25 mg T2MC (%)	0.5 mg T2MC (%)	1 mg T2MC (%)	2 mg T2MC (%)	4 mg T2MC (%)	PHBA control (4mg/mL)
0	40.9 ± 0.024	44.7 ± 0.006	47.0 ± 0.011	55.1 ± 0.001	55.4 ± 0.001	67.6 ± 0.004
2	56.1 ± 0.001	58.0 ± 0.001	61.4 ± 0.001	60.8 ± 0.001	57.7 ± 0.002	61.7 ± 0.007
4	58.5 ± 0.001	60.5 ± 0.001	64.6 ± 0.001	60.6 ± 0.002	57.4 ± 0.001	57.9 ± 0.002
6	59.1 ± 0.001	61.1 ± 0.001	65.4 ± 0.001	60.7 ± 0.002	56.5 ± 0.001	56.9 ± 0.001
8	59.5 ± 0.001	61.7 ± 0.001	66.2 ± 0.001	60.5 ± 0.002	64.6 ± 0.015*	59.9 ± 0.013
10	60.3 ± 0.001	62.5 ± 0.001	67.0 ± 0.001	61.3 ± 0.003	100 ± 0.035*	87.0 ± 0.018
12	60.3 ± 0.001	62.8 ± 0.001	67.4 ± 0.001	63.3 ± 0.001	100 ± 0.007*	97.1 ± 0.003
14	60.3 ± 0.001	62.9± 0.001	67.5 ± 0.001	68.1 ± 0.002	100 ± 0.001*	96.1 ± 0.01
16	61.5 ± 0.001	63.3 ± 0.001	68.7 ± 0.001	79.5 ± 0.012	100 ± 0.001*	99.9 ± 0.007
18	61.5 ± 0.001	63.6 ± 0.001	69.3 ± 0.001	96.2 ± 0.018	100 ± 0.002	100 ± 0.017
20	61.9 ± 0.001	64.1 ± 0.001	69.9 ± 0.001	100 ± 0.016	100 ± 0.001	100 ± 0.008
22	61.9 ± 0.001	64.1 ± 0.007	70.4 ± 0.001	100 ± 0.005	100 ± 0.001	100 ± 0.009
24	63.2 ± 0.001	65.2 ± 0.003	71.1 ± 0.001	100 ± 0.001	100 ± 0.001	100 ± 0.004
26	64.4 ± 0.001	66.5 ± 0.007	72.4 ± 0.001	100 ± 0.001	100 ± 0.001	100 ± 0.006
28	65.2 ± 0.001	67.4 ± 0.007	72.8 ± 0.001	100 ± 0.001	100 ± 0.002	100 ± 0.004
30	65.9 ± 0.001	68.1 ± 0.001	73.9 ± 0.001	100 ± 0.001	100 ± 0.001	100 ± 0.003

PHBA: para hydroxybenzoic acid, positive control.- statistically different ($p<0.05$) compared with the PHBA at same concentration.*

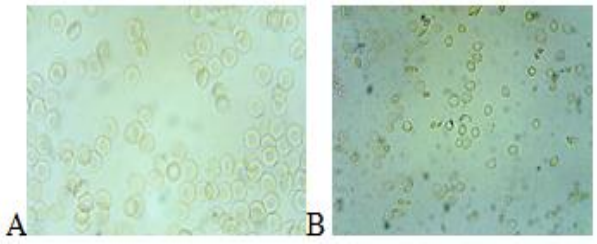

Fig 2. A) Representative slide showing blood film of Hb AA blood cells and **B)** showing the blood film of an Hb SS blood cells in steady state. (Magnification x400**)**

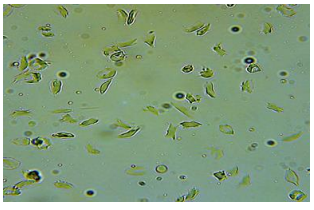

Fig 3. Photomicrograph **of** sickled Hb SS blood cells after deoxygenation with nitrogen gas (Negative control). Mniagfication x400)

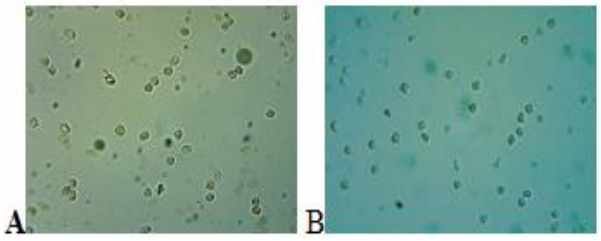

Fig 4. A) Photomicrograph of the inhibitory activity (95.40 ± 0.15 %) of T2MC and **B**) the reversal activity (95.60 ± 0.28 %) of T2MC (Magnification x400)

Comparing T2MC with PHBA (positive control) at 4 mg/mL, both gave 100% inhibition at 10 and 18 minutes, respectively, with significantly different ($p<0.05$) inhibitory properties. T2MC acts by preventing Hb S polymerization. The antisickling potential of any material can be measured by its ability to interfere with three different stages of sickling process. Antisickling agents may modify disease at the sickle gene level by preventing dehydration of red cells

(ion exchange through the cell membrane), by preventing Hb S polymerization or by increasing the production of Hb F (Dash *et al.*, 2013).

An important chemotherapeutic goal of intervention is the development of chemical modifiers that would minimize the tendency of deoxy-Hb S to polymerize. Drugs such as hydroxyurea and 2-imidazolines, interfere with and disrupt the contact point that promotes polymerization of deoxy-Hb S molecules (Charache *et al.*, 1995). The nature and mode of interaction between biochemical compounds and contact points of deoxy-Hb S molecules, as well as the erythrocyte membrane structural components are crucial determinants in their ability to retard/inhibit polymerization of deoxy-Hb S molecules (Oyewole *et al.*, 2008; Chikezie *et al.*, 2011). Anthocyanins, tannins and amino acids (aspartate, tyrosine and arginine), are phytochemicals implicated in the inhibition of the polymerization of deoxy-Hb S molecule (Mpiana *et al.*, 2010), and these were reported to be present in the extracts of MO, CA and TO (Fasuyi 2006; Mordi and Akanji 2012; Ojiako, 2014). These might be responsible for the stabilizing of the oxy-state of Hb S molecules that result into increase oxygen/hemoglobin affinity and cause reduction in the amount and rate of Hb S polymerization. These observations were obvious reflections of the ability of T2MC to bind and shield the contact points of Hb S monomers required for polymerization. The inhibition of deoxy-Hb S polymerization by T2MC combination and the PHBA (positive control) was dose and time dependent. This conforms with earlier reports of Oyewole *et al.* (2008).

3.2. Percentage stability of sickle RBC membrane

T2MC exhibited concentration dependent ability to protect the membrane of sickle erythrocytes significantly ($p<0.05$) than the standard drug, Ibuprofen (Figure 5).

The cell membrane of the SS RBCs has been targeted for therapeutic developments. One approach involves improving the hemo-rheology of SS RBCs in order to reduce their abnormal adhesion to vascular endothelial cells, inflammation and nitric oxide scavenging. T2MC exhibited significantly higher ($p<0.05$), concentration dependent ability to protect the membrane of SS RBCs than Ibuprofen, the standard drug (Figure 5).

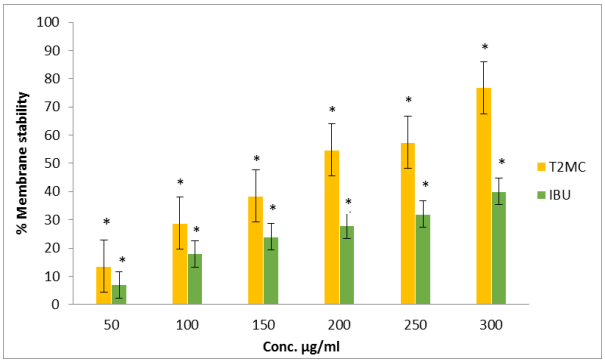

Fig 5. Membrane stability activities of T2MC and Ibuprofen control.
Key: IBU- Ibuprofen (positive control); * The mean difference is significant ($P<0.05$) compared with the control.

T2MC serves as a potential treatment that would promote elasticity and increase deformability of the RBC membrane, which could possibly improve the hemo-rheological properties of the RBCs of individuals with SCD (Athanassiou *et al.*, 2006). The loss of these properties in cells result in their inability to deliver oxygen to the tissues, leading to premature removal from circulation by the spleen, hence anemia (Delaunay, 2007). The membrane stabilizing activities of T2MC is also likely aided by the presence of flavonoids, tannins, and saponins in the ethanol leave extracts of the plants which have the ability to bind cations and stabilize cell membranes (Pathak *et al.*, 1991; El-Shabrany *et al.*, 1997; Awoyinka and Balogun, 2007).

3.3. Red cell fractionation assay results

The discontinuous density gradient provided sufficient RBCs in each fraction (F1, F2, F3, and F4) according to their densities.Hb AA blood samples layered on the discontinuous density gradient produced F1, F2 and F3 layers without the densest cell layer (F4). Untreated Hb SS blood, however, produced four fractions with F1 containing the reticulocytes which are the least dense cells, and F4 the densest (rigid) cells. The severely dehydrated dense fraction (F4) of sickle cells is known to have undergone various biochemical alterations, therefore this fraction contains most of the irreversibly sickled and most dense cells.

On treatment of Hb SS RBCs with T2MC and Ciklavit® (positive control), there was significant reduction in dense cell population of the F4 fraction. The percentage RBCs in the F4 layer of Hb SS blood treated with T2MCwas significantly lower (at $p<0.05$) than the corresponding Hb SS negative control (Table 3).

Table 3. Percent RBC count of Hb S blood before and after treatment with T2MC and control (Ciklavit®).

Fractions	% RBC count (Hb SS blood neat)	% RBC count (Hb SS blood + T2MC)	% RBC count (Hb SS blood + Ciklavit®)
F2	16.86 ± 0.52	16.97 ± 0.41	19.47 ± 0.14
F3	31.41 ± 0.60*	49.52 ± 0.43*	44.83 ± 0.41*
F4	51.73 ±1.29*	33.51 ± 0.86*	35.70 ± 0.16*

F2 contained young but mature red cells with the lowest density; F3 contained cells of intermediate age and density; F4 contained the densest cells. n=3; values are presented as ± SEM (standard error of mean).
**- Statistically different (p<0.05) compared with the Hb SS blood neat.*

The percentage change in the RBC count of cells treated with T2MC is not significantly different from that of Ciklavit® (Table 4). In SCD, increased cell density causes a reduction in RBC deformability. Dense RBCs that include a variable percentage of irreversibly sickled cells become rigid and cause decreased deformability, thus increasing the tendency to occlude small vessels. Additionally, the intracellular concentration of deoxy Hb S predisposes the sickling of RBCs, therefore the status of cellular hydration, which is also a function of Hb S concentration, plays a critical role in the pathological process (Eaton and Hofrichter, 1990). Messmann *et al*. (1990) reported that the membranes of the densest cells were rigid and they are less deformable than membranes from control cells or normally hydrated sickle cells.

Table 4. The percent change in the percentage of Hb SS red blood cells in fraction (F) 4 treated with T2MC and Ciklavit®.

Extracts/ control	Mean % Change in cell count of F4 fraction
T2MC	18.22 ± 0.86*
Ciklavit®	16.03 ± 0.16*

n=3; Values are presented as ± SEM (standard error of mean).-Significantly different (p<0.05) from Ciklavit® control***.**

T2MC significantly reduced the percentage RBCs in the densest F4 layer and increased the quantity of RBCs in the less dense F3 layer of the discontinuous gradient (Table 3). This implies that the administration of T2MC or the positive control Ciklavit® rehydrated and reversed dense cells (Table 4). The decrease in the number of dense SS RBCs is an indication that the cells have improved state of hydration and increased deformability. These changes will prevent polymerization and ultimately sickling.

3.4. Regulation of genes by T2MC

T2MC down regulated the expression of rKCCN-1, rKCCN-4 and Ca-K Channel genes and upregulated the G6PD genes in normal Wistar rats (Figures 6-9).T2MC exerted its antisickling property on Hb SS RBCs by down-regulating the Gardos' channel genes KCCN-4, and shutting down the expression of the KCCN-1 and Ca-K channel genes at 50 mg/kg (Figures 6a-c).Inhibition of KCNN-4is a prime target based on blocking ion transport pathways mediating ion efflux in SCA (Brugnara *et al.*, 1993). Various studies including *in vitro*, *in vivo* in transgenic sickle mice, and in patients have shown that prevention of sickle cell dehydration via the Gardos channel (Ca^{2+} activated K^+ channel)is a feasible strategy which can be achieved by using specific blockers of ion transport pathways to mediate potassium loss from the sickle erythrocyte (Brugnara, 2001). Clotrimazole, ICA-17043, Nitrendipine, Cetiedil, charybdotoxin (ChTx) and maurotoxin (MaTx) are reported potent Gárdoschannel blockers, however, none have been clinically useful in SCD, therefore phytomedicine represents a cost-effective therapeutic potential for drug candidates as lead in the management of SCD (Imaga, 2013). T2MC is a potent Gardos channel blocker which would prevent potassium efflux and hence dehydration of cells.

Erythropoietin (EPO) is a glycoprotein hormone that induces RBC production, and acts by binding to its specific receptor on the surface of erythroid progenitor cells to stimulate cell survival, proliferation and differentiation. In our *in vivo* study, the mRNA profiling of erythropoietin receptor and its cognate ligand, erythropoietin, of folic acid at 50 mg/kg showed the upregulation of both EPO and EPO-R. Folic acid induced erythropoiesis more significantly than the control (Figures 7a-b) thus confirming its well-known effect on erythropoiesis. T2MC at 50 and 100 mg/ kg, however, down regulated the EPO and EPO-R genes, indicating that T2MC did not exert its antisickling property by increasing erythropoiesis. This observation was corroborated by the non-significant expression of the cell cycle gene p-21 by T2MC (Figure 8a-b). There

was significant upregulation of the antioxidant genes (GPX-1, GSR, CAT and G6PD) by folic acid at 50 mg/kg (Figures 9a-d) while T2MC upregulated the expression of only the G6PD enzyme, thus suggesting its effect on the protection of the deficient RBCs.

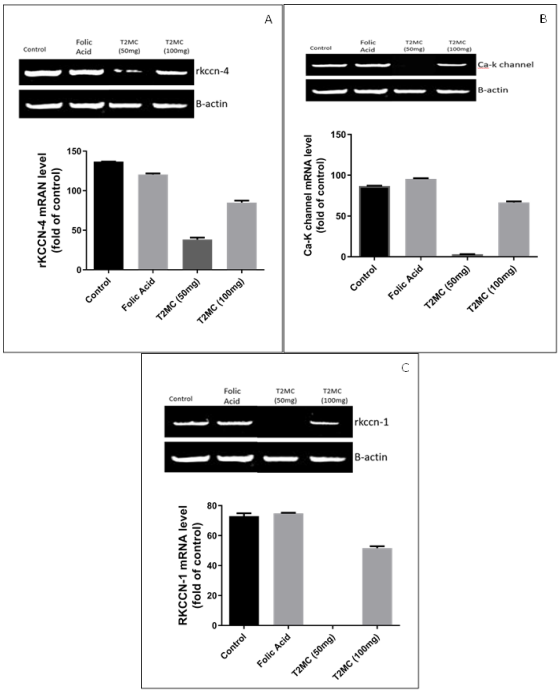

Figure 6. The down regulation of the Gardos channel genes (**A:** rKCCN-4, **B:** Ca-k channel, and **C:** rKCCN-1) by T2MC.

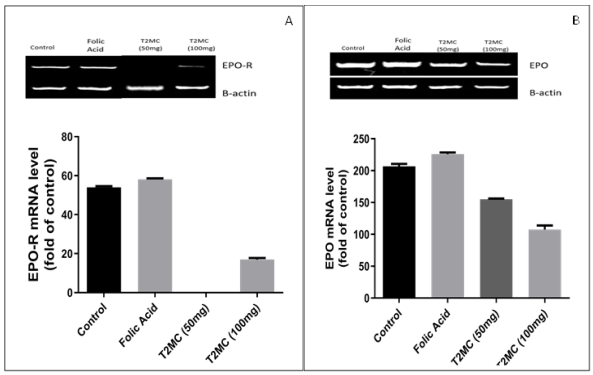

Figure 7. The effect of T2MC and Folic acid on the expression of **A**) EPO-R and **B**) EPO genes.

The G6PD enzyme offers some protection on RBCs and reduces the incidence of anemia in SCD (Benkerrou *et al.*, 2013), from oxidative damage by supplying reducing energy to them, and maintaining the level of reduced co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH).

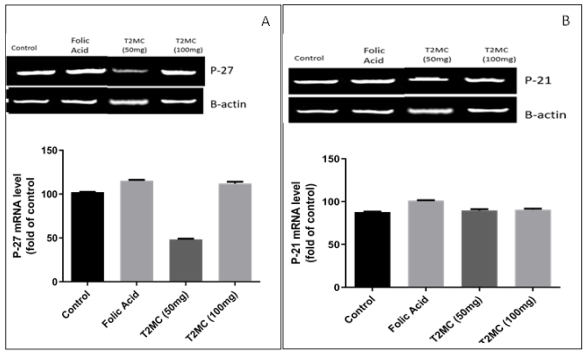

Figure 8. The effect of T2MC and folic acid on the expression of the cell cycle genes, **A**) p21 and **B**) p27.

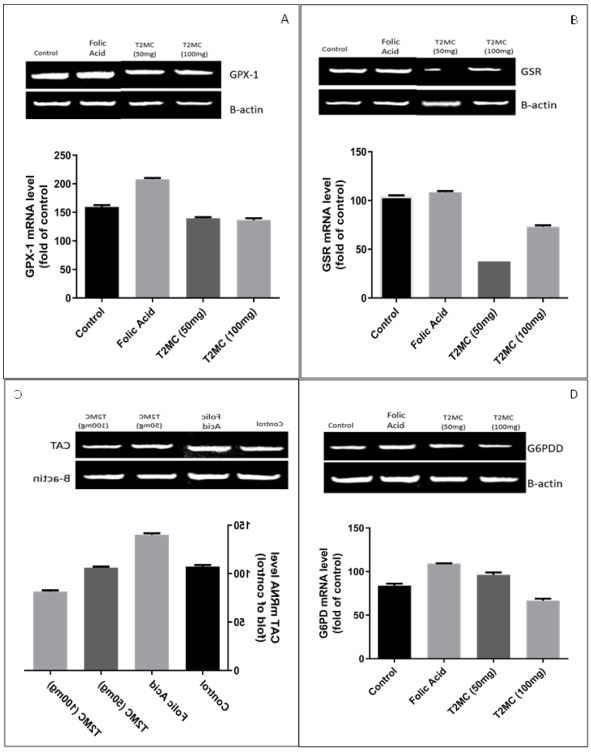

Figure 9 A-D: The effects of folic acid and T2MC on the expression of the antioxidant genes (GPX-1, GSR, CAT, and G6PD).

4. Conclusion

The effect of T2MC on SS RBC membrane, percentage dense cells, and inhibition of polymerization, as well as the *in vivo* animal studies established the antisickling property and the mechanism of action of T2MC polyherbal product, but more detailed studies are required**.**

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