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INVESTIGATION OF SELECTED HERBOMINERAL FORMULATION T-AYU-H & T-AYU-HM™ PREMIUM AS AN ANTISICKLING AGENT FOR SICKLE CELL ANEMIA

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Keywords:

Sickle cell anaemia, Herbomineral formuulation, Emmel's test, t-BOOH, T-AYU-H and T-AYU-HM™ Premium

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ABSTRACT: Sickle cell anaemia is a genetic blood disorder that is caused by a mutation in the gene that encoded *β*-globin of haemoglobin, which results as the presence of abnormal haemoglobin HbS in the RBC. Presently, there is no universal cure for sickle cell anaemia Herbomineral formulations were standardized for organoleptic properties (colour, odour and taste), physiochemical properties, total ash value, moisture content, pH of the solution, to find effective agent in which we investigated the antisickling potential of T-AYU- HM Premium, a novel Herbomineral formulation. *In-vitro* sickling of homozygous sickle cells upon deoxygenation (Emmel's Test) showed dose dependent inhibition of sickling in presence. T-AYU-H and T-AYU-HM Premium (25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml). Mechanistic evaluations were performed such as, t-BOOH induced oxidative haemolysis, and formation of met-haemoglobin was significantly decreased in dose dependent manner in the presence of 50 µg/ml, 100 µg/ml and 500 µg/ml of T-AYU-H and T-AYU-HM Premium. Membrane stabilization activity by osmotic fragility test showed significant dose dependent increased stabilization in the presence of 50 µg/ml, 100 µg/ml and 500 µg/ml of T-AYU-H and T-AYU-HM Premium. The T-AYU-H and T-AYU-HM Premium will provide improvement of the therapeutic profile and lesser side effects compare to available synthetic treatment such as Hydroxyurea and Decitabine, and chances of blood transfusions will also be decreased. Formulations are much more affordable compared to available cost of management of disease for sickle cell anemic patients.

INTRODUCTION: Sickle cell anaemia is an autosomal recessive genetic hematologic disorder that results from point mutation in *β*-globin gene, a major subunit of hemoglobin. A single nucleotide substitution (GAG - GTG) in sixth codon of the β-globin gene results in substitution of valine for glutamic acid.

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This mutation causes low affinity of hemoglobin for oxygen, thus mutant hemoglobin polymerizes into a gel or into fibers causing decrease in red cell deformability. Hemoglobin S polymerization causes change of erythrocytes from their normal globular form into sickle shape [1].

Sickle cell anaemia is a vasculopathy in which the deoxygenation-induced polymerization of HbS causes erythrocyte damage this triggers a cascade of RBC dehydration, hemolysis and vaso-occlusion. The pathology of sickle cell disease (SCD) is complicated, in addition to moderate to severe anemia; it often includes infarction of the spleen during childhood, which results in

diminished immune function. Growth and development are delayed, and the ability to produce offspring is often lessened. Bone and eye disorders may occur. Painful episodes punctuate the course of the disease in many patients, and they may be characterized by unbearable pain in the legs and arms, back, chest, or abdomen. Also affected are the cardiovascular, pulmonary, and renal systems.

Haemoglobinopathies, mainly sickle-cell anaemia and thalassaemias, are globally widespread. About 5% of the world's population carries genes responsible for haemoglobinopathies. (WHO Report, April 24, 2006). Based on the prevalence rates of sickle cell haemoglobin, it was estimated that there were over 50,00,000 persons carriers and two lakhs homozygous sickle cell anemia cases among tribes alone in India. Orissa is the most affected *i.e.* 12% population suffers from sickle cell haemoglobinopathies. The other states affected in decreasing order are Madhya Pradesh (including Chhattisgarh), Tamil Nadu, Andhra Pradesh, Assam, Maharashtra, and Gujarat. The sickle cell disorder is common mostly in scheduled tribes.

In the present investigation of Herbomineral formulations, comprising of a polyherbal composition of recommended Ayurvedic plants along with a few essential mineral elements selected on the basis of scientific evidence on their mechanism of action in the human body for the treatment of sickle cell anemia.

MATERIAL AND MATHODS:

Polyherbal and Herbo - Mineral (HM) Formulations: Polyherbal (T-AYU-H) and Herbomineral formulations (T-AYU-HM Premium, oral tablet 300 mg/BD) were used for the Pharmacological screening and evaluation of activity for treatment of sickle cell anaemia. The protocol was designed as per ethical guidelines and obtained the approval from ethics committee (code no: 10/12/01-MNC) in 2010.

T-AYU-H Tablet:

Formulation Type: Polyherbal

Composition: Each T-AYU-H tablet (300 mg) contains: *Aloe vera* (50 mg), *Filamlethurs emblica* (50 mg), *Terminala chebula* (25 mg), *Zingiber officinale* (25 mg), *Asparagus racemosus* (25 mg), *Punica granatum* (12.5 mg), *Myristica fragrans* (12.5 mg), *Piper longum* (25 mg), *Tinospora cordifolia* (12.5 mg), *Leptadinia reticlata* (12.5 mg), *Plambekjinilika* (12.5 mg).

Manufactured By: ATBU Harita Pharmaceuticals Pvt Ltd., Vyara.

T-AYU-HM™ Premium Tablet:

Formulation Type: Herbomineral

Composition: Each T-AYU-HM Premium tablet (300 mg) contains: Calyx of Mica (25 mg), Calyx of iron (12.5 mg), *Terminala chebula* (25 mg), *Zingiber officinale* (25 mg), *Asparagus racemosus* (25 mg), Punica granatum (12.5 mg), *Myristica fragrans* (25 mg), *Piper longum* (37.5 mg), *Tinospora cordifolia* (37.5 mg), *Leptadinia reticlata* (37.5 mg).

Manufactured by: ATBU Harita Pharmaceuticals Pvt Ltd., Vyara.

All the above formulations were supplied by Dr. Atul Desai, Dhanvantari Clinic, Vyara - 394850.

Blood Samples: Whole blood samples from homozygous sickle cell patients (Hb SS) were collected at Dhanvantri Clinic, Vyara, Surat drawn into sterile Vaccuete tubes, stored at 2-8 ºC and used within a period of 2 weeks.

***In-vitro* Antisickling Activity Models:**

Preparation of Erythrocyte Suspension: The blood samples were washed repeatedly by using centrifugation thrice at 1500 rpm for 10 min with phosphate buffer saline (PBS), at pH 7.4, in order to remove buffy coat and dense cells. For control, RBCs mixed with PBS and for treatment of test, RBCs were put in contact with test formulations *i.e*. T-AYU-H and T-AYU-HM Premium containing different concentrations *viz*. 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml. 10 mM Vanillin was used as standard antisickling agent. The morphological changes in sickle erythrocytes after deoxygenating were evaluated using experimental models described below:

Emmel's Test (anaerobic condition induced deoxygenation): Emmel's test protocol as described by Mpiana [9] followed. Erythrocyte suspension put on slide and hermetically covered

by cover glass using vacuum grease. In order to ensure complete anaerobic conditions the slides were placed in vacuum desiccator and then transferred to incubator (37 ºC) for 24 h. The slides were observed for the presence of sickle cells and count using photomicroscope with magnification at 45x, digitalization of images with DTV250 software, Morphological analysis of erythrocytes was performed in duplicate, the RBCs with star shape or elongated shape were counted as sickle cells, while circular shape RBCs were counted as Normal cells.

Sodium Metabisulphite Induced Deoxygenation: [9] Erythrocyte suspension was deoxygenated with 2% sodium metabisulphite ($Na_2S_2O_5$) for 5 min at 37 ºC. Then the cells were placed under anaerobic conditions between glass cover slips [8]. The slides were observed after 40 min for the presence of sickle cells and count using photomicroscope with magnification at 45x, digitalization of images with DTV250 software, morphological analysis of erythrocytes was performed in duplicate, the RBCs with star shape or elongated shape were counted as sickle cells, while circular shape RBCs were counted as normal cells.

***In-vitro* Mechanistic Evaluation of Antisickling Activity:**

Membrane Stabilizing Activity by using Osmotic Fragility Model: The fragility of RBC was determined by placing the cells in graded series of hypotonic saline solutions buffered at pH 7.4 with 150 mM phosphate. Concentrations ranging from 0.1% to 0.9% NaCl were made. A 0.01 ml aliquot of blood sample was added to 1 ml of the various hypotonic solutions, and immediately mixed by inverting several times. The tubes were incubated at 37 ºC for 1 h. The contents were re-mixed and centrifuged for 5 min at 1500 g. The absorbance of the supernatant was read at 540 nm using 9.0 g/l NaCl tube as blank. Each blood sample was used twice and the average taken. For the effect of the test formulations, 0.1 ml different concentrations of test formulations were added to 0.9 ml of NaCl solutions. A 0.01 ml aliquot of blood sample was added and the mixture treated as described earlier. The mean corpuscular fragility (which is the concentration of saline causing 50% haemolysis of the erythrocytes) was obtained from a plot of % lyses against NaCl concentration (g/l). [9]

***t*-BOOH Induced Haemolysis of Sickle Erythrocyte:** [2] Blood samples were washed thrice by consecutive centrifugations in phosphate buffer saline (PBS) pH 7.4 to remove plasma and the buffy coat. Then RBCs were added to PBS containing 0.5 mM *t*-BOOH. RBC oxidation was carried out by incubating RBC suspensions (HT 2%) in 0.5 mM *t*-BOOH at 37 ºC, in the absence or in the presence of different concentrations of test formulation *i.e.* 50 µg/ml, 100 µg/ml and 500 µg/ml. *t*-BOOH was added to the erythrocyte suspension as ethanol solution (not more than 0.5 of the total incubation volume).

The extent of the time dependent haemolysis was determined as follows. A volume of the incubation mixture at any given incubation time 0, 15, 30, 60, 90, 120 min and 12 h was diluted with PBS and centrifuged at 5000 g for 2 min to precipitate the cells. Absorbance of the supernatant was then evaluated at 540 nm and 630 nm.

Similarly, a volume of the same incubation mixture was treated with5 mM Sodium phosphate buffer, pH 7.4 (hypotonic buffer) and exposed to an ultrasonic bath for 1 min to yield complete haemolysis. After a centrifugation at 5000 g for 2 min the absorbance of the supernatant was evaluated at 540 nm and 630 nm. The percentage of haemolysis was calculated from the ratio of the absorbance.

***In-vivo* Animal Model:** Stasis induced venous thrombosis model in SD rat female Sprague dawley rats weighting 200-300 g were selected and randomized into 11 group (as per mention below) each having 5 rats. The rats were treated for 5 days, and dose was administered once a day with vehicle *i.e.* 0.5% CMC. Test formulations *i.e.* T-AYU-H and T-AYU-HM Premium having different dose (100, 300, 500 mg/kg p.o) and as standard drug Acetyl salicylic acid (ASA 30 mg/kg p.p.). One hour after the last administration animals were anesthetized by intraperitoneal injection of 1.3 g/kg urethane.

The inferior vena cava was exposed through a midline incision from the renal vein to the iliac bifurcation and $FeCl_3$ saturated Whatman filter paper no. 1 was placed to inferior vena cava for 3 min. $FeCl_3$ paper was removed and venous stasis

was induced by tying the knot of same distance of $FeCl_3$ paper. The animal was then kept for 30 min for venous stasis and body temperature was maintained. Finally, remove the tie parts of inferior vena cava, cleaned and weight of vein with and without thrombus was taken to find out weight of thrombus formed in the vein [6].

Statistical Analysis: Experiment was carried out in duplicate using RBC suspension from three subjects. The results were expressed as Means ± SD and were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. Differences at $P<0.05$ were considered significant compared to control.

Standardization of Herbomineral Formulation:

TABLE 1: DESCRIPTION OF THE FORMULATIONS AND EXTRACTS USED IN THE FORMULATIONS

Samples	Color	Odour	Taste
Terminalia chebula	Brown	Characteristic	Astringent
Zingiber officinale	Light brown	Characteristic	Pungent
Asparagus racemosus	Brown	Characteristic	Slightly bitter
Myristica fragrans	Cream	Aromatic odor	Characteristic
Tinospora cordifolia	Brown	Characteristic	Bitter
Leptadinia reticulata	Brown	Characteristic	Astringent
Punica granatum	Brown	Characteristic	Astringent
Aloe vera	Brown	Characteristic	Astringent
Piper longum	Greenish	Characteristic	Pungent
T-AYU-HM Premium	Dark blackish	Characteristic	Bitter
T-AYU-H	Dark reddish	Characteristic	Slightly bitter

TABLE 2: PERCENT OF ALCOHOL AND WATER SOLUBLE EXTRACTIVES OF FORMULATIONS AND EXTRACTS USED IN THE FORMULATIONS

Samples	Alcohol soluble (%) n=6 ± SEM	Water soluble (%) n=6 ± SEM
Terminalia chebula	76.22 ± 0.62	78.47 ± 0.42
Zingiber officinale	21.61 ± 0.42	16.25 ± 0.76
Asparagus racemosus	76.98 ± 0.96	85.07 ± 0.55
Myristica fragrans	21.61 ± 0.62	16.77 ± 0.51
Tinospora cordifolia	78.56 ± 1.85	81.24 ± 0.68
Leptadinia reticulata	76.04 ± 0.56	74.76 ± 0.43
Punica granatum	72.58 ± 0.56	81.25 ± 0.41
Aloe vera	75.02 ± 0.84	80.24 ± 0.54
Piper longum	22.29 ± 0.41	16.42 ± 0.51
T-AYU-HM Premium	70.81 ± 2.10	64.67 ± 1.31
T-AYU-H	77.00 ± 0.42	65.33 ± 1.74

TABLE 3: PERCENT LOSS ON DRYING AND pH OF THE 1% w/v SOLUTION OF FORMULATIONS AND EXTRACTS USED IN THE FORMULATIONS

Samples	Loss on drying (%) n=6 ± SEM	pH 1% w/v solution
Terminalia chebula	3.85 ± 0.010	3.8
Zingiber officinale	6.72 ± 0.006	5.25
Asparagus racemosus	2.41 ± 0.010	4.98
Myristica fragrans	6.62 ± 0.006	4.85
Tinospora cordifolia	2.4 ± 0.001	4.96
Leptadinia reticulata	1.96 ± 0.012	5.73
Punica granatum	2.18 ± 0.018	4.28
Aloe vera	2.47 ± 0.017	4.87
Piper longum	6.08 ± 0.030	4.79
T-AYU-HM Premium	7.37 ± 0.021	5.02
T-AYU-H	7.81 ± 0.035	4.16

TABLE 4: PHOTOCHEMICAL EVALUATION OF FORMULATIONS FOR THE PRESENCE OF CHEMICAL CONSTITUENTS

Chemical constituents	T-AYU-H	T-AYU-HM Premium
Alkaloids	+	+
Proteins	+	+
Carbohydrates	+	+
Saponins	-	-
Glycosides	+	+
Flavonoids	+	+
Amino acids	+	+
Fixed oils	-	-
Essential oil	-	-
Tannins/Phenolic compounds	+	+

RESULTS:

Emmel's Test (*In-vitro* Antisickling Activity): Sickle cell anaemic blood samples caused significant increase in the sickling of RBCs to 67.94 ± 4.47% after incubation in anaerobic condition **Table 5**. Vanillin (10 mM) significantly decreased the sickling of RBCs to 24.07 ± 10.66% **Table 5**. The percentage inhibition of sickling in presence of vanillin (10 mM) was found to be significantly increased to 64.62 ± 15.41% **Table 6**.

TABLE 5: PERCENTAGE OF SICKLE CELLS AND INHIBITION OF SICKLE CELLS IN PRESENCE OF T-AYU-H USING EMMEL'S TEST

Treatment	% Sickle cells Mean ± SD	% Inhibition of sickling Mean ± SD
Control (PBS)	67.94 ± 4.47	0.00
10 mM Vanillin	24.07 ± 10.66*	64.62 ± 15.41*
25 µg/ml T-AYU-H	59.04 ± 3.55	12.97 ± 4.95*
50 µg/ml T-AYU-H	48.45 ± 7.52 *	28.44 ± 11.95*
100 µg/ml T AYU-H	36.87 ± 5.54*	45.87 ± 5.67*
500 µg/ml T-AYU-H	27.14 ± 5.81*	60.15 ± 7.71*

n=5, *p<0.05 as compared to control samples using one way ANOVA followed by Dunnett's Test

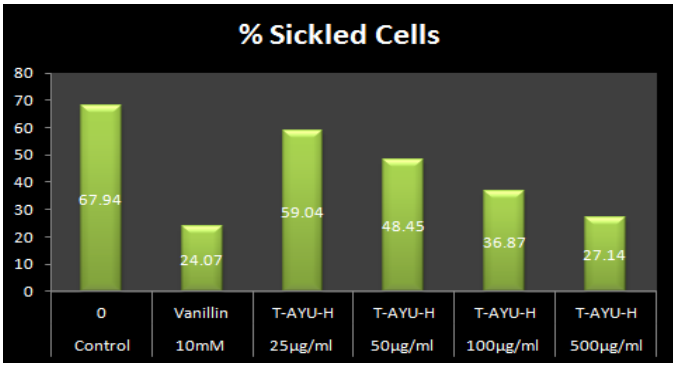


FIG. 1: PERCENTAGE OF SICKLED CELLS IN PRESENCE OF T-AYU-H

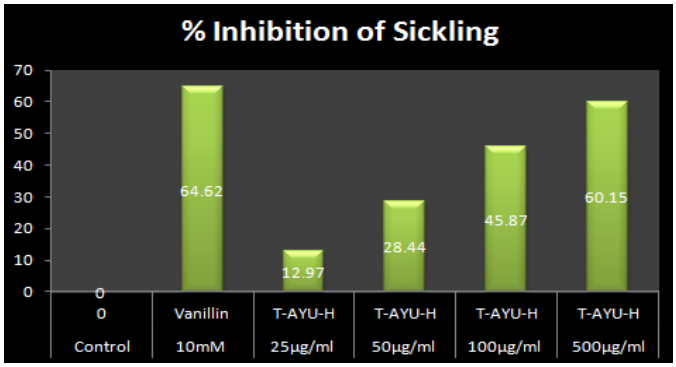
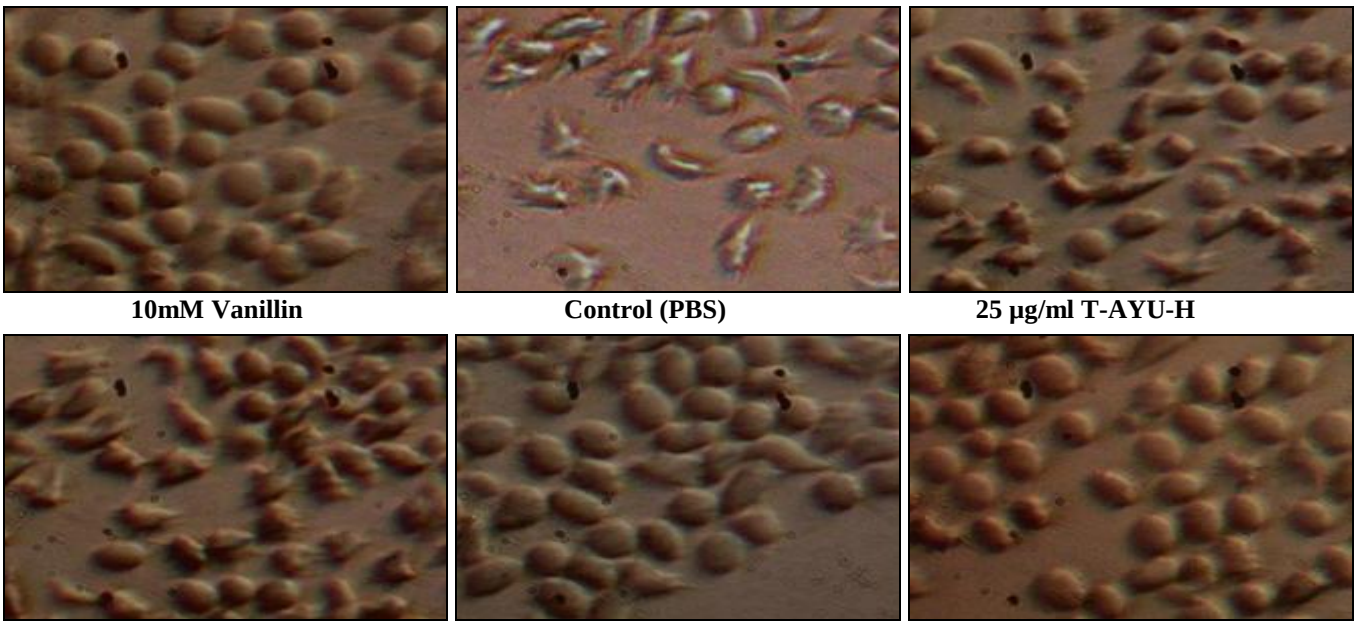


FIG. 2: PERCENTAGE INHIBITION OF SICKLED CELLS IN PRESENCE OF T-AYU-H

n=5, * p<0.05 as compared to control samples using one way ANOVA followed by Dunnett's Test

Effect of T-AYU-H in the Emmel's Test Protocol: In presence of different concentrations of T-AYU-H *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, respectively percentage of sickled cells were found to be 59.04 ± 3.55%, 48.45±7.52%, 36.87 ± 5.54% and 27.14 ± 5.81% **Table 5** and **Fig. 1**.

The percentage inhibition of sickling in presence of different concentrations of T-AYU-H *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, were found to be 12.97 ± 4.95%, 28.44 ± 11.95%, 45.87 ± 5.67% and 60.15 ± 7.71% respectively as compared to control **Table 5** and **Fig. 2**.



10mM Vanillin **Control (PBS)** **25 µg/ml T-AYU-H**

50 µg/ml T-AYU-H **100 µg/ml T-AYU-H** **500 µg/ml T-AYU-H**

FIG. 3: THE MORPHOLOGY OF SICKLE CELLS THAT WERE INCUBATED UNDER ANAEROBIC CONDITION IN THE ABSENCE OR PRESENCE OF VARIOUS CONCENTRATIONS OF T-AYU-H AND 10 mM VANILLIN FOR 24 h

Effect of T-AYU-HM Premium in the Emmel's Test Protocol: In presence of different concentrations of T-AYU-HM Premium *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, respectively percentage of sickled cells were found to be 55.22 ± 4.66% , 44.28 ± 5.77%, 39.34 ± 5.90% and 27.41 ± 8.12% **Table 6** and **Fig. 4**. The percentage inhibition of sickling in presence of different concentrations of T-AYU-HM Premium *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, were found to be 18.32 ± 10.11%, 34.51 ± 10.21%, 41.83 ± 10.33% and 59.85 ± 11.36% respectively as compared to control **Table 6** and **Fig. 5**.

TABLE 6: PERCENTAGE OF SICKLE CELLS AND INHIBITION OF SICKLE CELLS IN PRESENCE OF T-AYU-HM PREMIUM USING EMMEL'S TEST

Treatment	% Sickle Cells Mean ± SD	% Inhibition of sickling Mean ± SD
Control (PBS)	67.94 ± 4.47	0.00
10 mM Vanillin	24.07 ± 10.66	64.62 ±15.41
25 µg/ml HMP	55.22 ± 4.66	18.32 ± 10.11
50 µg/ml HMP	44.28 ± 5.77	34.51 ± 10.21
100 µg/ml HMP	39.34 ± 5.90	41.83 ± 10.33
500 µg/ml HMP	27.41 ± 8.12	59.85 ± 11.36

n=5, *p<0.05 as compared to control samples using one way ANOVA followed by Dunnett's Test

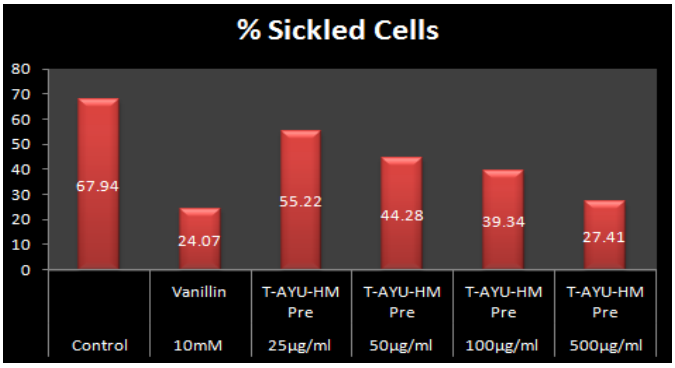


FIG. 4: PERCENT OF SICKLED CELLS IN PRESENCE OF T-AYU-HM PREMIUM

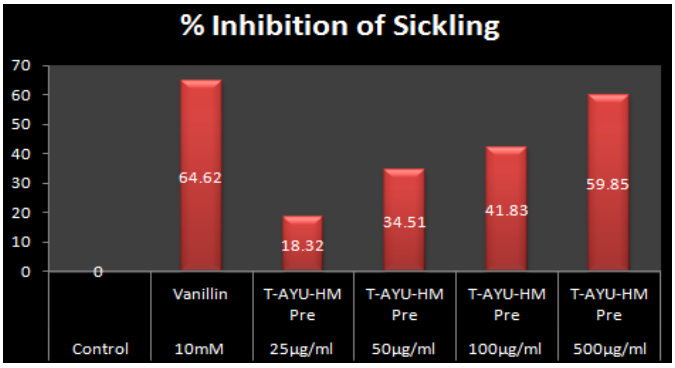


FIG. 5: PERCENTAGE INHIBITION OF SICKLED CELLS IN PRESENCE OF T-AYU-HM PREMIUM

n=5, * p<0.05 as compared to control samples using one way ANOVA followed by Dunnett's Test

Sodium Metabisulphite Test (*In-vitro* Anti-sickling Activity): Sodium metabisulphite ($Na_2S_2O_4$) Test *i.e.* incubation of $Na_2S_2O_4$ of drug solution with the sickle cell anaemic patient blood samples which caused significant increase in the sickling of RBC to 70.84 ± 8.18% **Table 7**. While in the presence of 10 mM Vanillin sickle cells were found to be significantly decreased to 24.14 ± 3.64%. The percentage inhibition of sickling in the presence of 10 mM Vanillin as standard was found to be significantly increased to 65.92 ± 6.31% **Table 7**.

TABLE 7: PERCENTAGE OF SICKLE CELLS AND INHIBITION OF SICKLE CELLS IN PRESENCE OF T-AYU-H USING SODIUM METABISULPHITE TEST

Treatment	% Sickle Cells Mean ± SD	% Inhibition of sickling Mean ± SD
Control (PBS)	70.84 ± 8.18	0.00
10 mM Vanillin	24.14 ± 3.64	65.92 ± 6.31
25 µg/ml T-AYU-H	53.74 ± 12.11	24.13 ± 11.21
50 µg/ml T-AYU-H	42.89 ± 5.52	39.45 ± 5.87
100 µg/ml T AYU-H	35.58 ± 5.91	47.77 ± 4.71
500 µg/ml T-AYU-H	26.66 ± 6.80	62.37 ± 6.99

n=5, * p<0.05 as compared to control samples

TABLE 8: EFFECT OF T-AYU-H ON % HAEMOLYSIS OF SICKLE ERYTHROCYTES

Time	% Haemolysis			
	Control	50 µg/ml T-AYU-H	100 µg/ml T-AYU-H	500 µg/ml T-AYU-H
0 Min	11.84 ± 0.74	8.77 ± 1.44	11.61 ± 3.61	8.95 ± 2.80
15 Min	13.60 ± 4.92	9.29 ± 3.12	8.70 ± 4.84	5.19 ± 5.68
30 Min	21.02 ± 6.33	16.64 ± 4.85	12.78 ± 3.98	6.95 ± 4.47
60 Min	25.57 ± 1.20	17.49 ± 5.45*	17.39 ± 2.81*	7.94 ± 4.82*
90 Min	45.18 ± 3.84	36.29 ± 2.91*	34.01 ± 3.26*	29.75 ± 0.98*
120 Min	60.58 ± 1.98	44.33 ± 3.66*	40.16 ± 3.18*	29.48 ± 3.44*
12 Hours	79.66 ± 3.71	61.91 ± 6.04*	46.51 ± 5.88*	28.39 ± 3.04*

n=5, * p<0.05 as compared to control samples using one-way ANOVA followed by Dunnett's Test

Effect of T-AYU-H in the Sodium Meta-bisulphite Test Protocol: In presence of different concentrations of T-AYU-H *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, respectively percentage of sickled cells were found to be 53.74 ± 12.11 %, 42.89 ± 5.72%, 35.58 ± 5.91% and 26.66 ± 6.8% **Table 7**. The percentage inhibition of sickling in presence of different concentrations of T-AYU-H *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, were found to be 24.13 ± 11.21%, 39.45 ± 5.87%, 49.77 ± 4.71% and 62.37 ± 6.99% respectively as compared to control **Table 7**.

Effect of T-AYU-HM Premium in the Sodium Metabisulphite Test Protocol*:* In presence of different concentrations of T-AYU-HM Premium *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, respectively percentage of sickled cells were found to be 50.47 ± 11.6% , 41.83 ± 6.54%, 33.0 ± 7.57% and 28.91 ± 7.12 % **Table 11**.

The percentage inhibition of sickling in presence of different concentrations of T-AYU-HM Premium *i.e.* 25 µg/ml, 50 µg/ml, 100 µg/ml and 500 µg/ml, were found to be 28.91 ± 12.19%, 41.01 ± 5.31%, 53.5 ± 8.40% and 59.32 ± 8.24% respectively as compared to control **Table 11**.

***t*-BOOH Induced Haemolysis of Sickle Erythrocyte:**

Effect of T-AYU-H on % Haemolysis of Sickle Erythrocyte: Dose and time dependent decrease in haemolysis was observed with increasing concentration of formulation. **Table 12** shows the % haemolysis for treatment of T-AYU-H at concentrations 50, 100 and 500 µg/ml at 60 min, 90 min, 120 min and 12 h **Table 8**.

Effect of T-AYU-H on % Met-Haemoglobin of Sickle Erythrocyte: Dose and time dependent decrease in methaemoglobin formation was observed with increasing concentration of formulation. **Table 12** shows the % haemolysis for treatment of T-AYU-H at concentrations 50, 100 and 500 µg/ml at 60 min, 90 min, 120 min and 12 h **Table 9**.

TABLE 9: EFFECT OF T-AYU-H ON % MET-HAEMOGLOBIN OF SICKLE ERYTHROCYTES

Time	% Met-haemoglobin			
	Control	50 µg/ml T-AYU-H	100 µg/ml T-AYU-H	500 µg/ml T-AYU-H
0 Min	13.01 ± 0.99	10.85 ± 1.35	10.50 ± 0.36	7.04 ± 1.94
15 Min	21.39 ± 2.74	13.08 ± 3.07	11.60 ± 4.16	5.66 ± 5.10
30 Min	27.83 ± 6.18	23.86 ± 8.81	17.98 ± 4.70	12.61 ± 1.63
60 Min	36.01 ± 7.50	26.73 ± 6.65	19.97 ± 3.00*	10.59 ± 4.77*
90 Min	57.36 ± 3.13	46.08 ± 6.02*	33.77 ± 7.38*	21.15 ± 3.65*
120 Min	63.87 ± 1.65	56.88 ± 5.66*	46.14 ± 5.03*	30.11 ± 2.78*
12 Hours	74.37 ± 6.85	63.42 ± 6.38*	52.59 ± 6.33*	30.39 ± 2.39*

n=5, * p<0.05 as compared to control samples using one-way ANOVA followed by Dunnett's Test

TABLE 10: EFFECT OF T-AYU-H ON MEMBRANE STABILIZATION ACTIVITY

Conc. of NaCl (%)	% Met-haemoglobin			
	Control	50 µg/ml T-AYU-H	100 µg/ml T-AYU-H	500 µg/ml T-AYU-H
0.90	14.11 ± 0.22	13.78 ± 0.23	13.43 ± 0.22	12.14 ± 0.20
0.80	14.65 ± 0.22	14.75 ± 0.24	14.04 ± 0.24	12.65 ± 0.21
0.70	17.04 ± 0.25	17.07 ± 0.26	15.53 ± 0.27	14.87 ± 0.24
0.60	32.99 ± 0.48	28.72 ± 0.42	25.89 ± 0.45	22.86 ± 0.36
0.55	50.61 ± 0.73	46.04 ± 0.67	41.47 ± 0.70	35.17 ± 0.55
0.50	69.11 ± 0.98	65.62 ± 0.94	62.02 ± 1.11	56.97 ± 0.92
0.40	85.59 ± 1.22	83.98 ± 1.22	80.26 ± 1.43	77.65 ± 1.25
0.30	92.81 ± 1.32	92.12 ± 1.32	91.23 ± 1.61	88.98 ± 1.42
0.10	96.50 ± 1.37	95.35 ± 1.36	93.36 ± 1.63	93.07 ± 1.48
0.00	100.00 ± 0.00	96.79 ± 1.37	94.73 ± 1.66	94.47 ± 1.50

TABLE 11: PERCENTAGE OF SICKLE CELLS AND INHIBITION OF SICKLE CELLS IN T- AYU-HM PREMIUM USING SODIUM METABISULPHITE TEST

Treatment	% Sickle Cells Mean ± SD	% Inhibition of sickling Mean ± SD
Control (PBS)	70.84 ± 8.18	0.00
10 mM Vanillin	24.14 ± 3.64	65.92 ± 6.31
25 µg/ml T-AYU-H	53.74 ± 12.11	24.13 ± 11.21
50 µg/ml T-AYU-H	42.89 ± 5.52	39.45 ± 5.87
100 µg/ml T AYU-H	35.58 ± 5.91	47.77 ± 4.71
500 µg/ml T-AYU-H	26.66 ± 6.80	62.37 ± 6.99

n=5, * p<0.05 as compared to control samples

Effect of T-AYU-HM Premium on % Haemolysis of Sickle Erythrocyte: Dose and time dependent decrease in haemolysis was observed with increasing concentration of formulation. **Table 12** shows the % haemolysis for treatment of T-AYU-HM Pre at concentrations 50, 100 and 500 µg/ml at 60 min, 90 min, 120 min and 12 h **Table 12**.

TABLE 12: EFFECT OF T-AYU-HM PREMIUM ON % HAEMOLYSIS OF SICKLE ERYTHROCYTES

Time	% Haemolysis			
	Control	50 µg/ml T-AYU-HM Pre	100 µg/ml T-AYU- HM Pre	500 µg/ml T-AYU- HM Pre
0 Min	11.84 ± 0.74	7.18 ± 1.36	10.42 ± 3.41	9.85 ± 2.65
15 Min	13.60 ± 4.92	7.66 ± 2.94	7.67 ± 4.57	6.30 ± 5.37
30 Min	21.02 ± 6.33	14.61 ± 4.59	11.53 ± 3.76	7.96 ± 4.22
60 Min	25.57 ± 1.20	15.41 ± 5.15*	15.88 ± 2.65*	8.90 ± 4.55*
90 Min	45.18 ± 3.84	33.18 ± 2.75*	31.58 ± 3.08*	29.50 ± 0.92*
120 Min	60.58 ± 1.98	40.77 ± 3.45*	37.40 ± 3.00*	29.25 ± 3.25*
12 Hours	79.66 ± 3.71	57.39 ± 5.70*	43.39 ± 5.55*	28.22 ± 2.87*

n=5, * p<0.05 as compared to control samples using one-way ANOVA followed by Dunnett's Test

Effect of T-AYU-HM Premium on % Met-Haemoglobin of Sickle Erythrocyte: Dose and time dependent decrease in methaemoglobin formation was observed with increasing concentration of formulation. **Table 12** shows the % haemolysis for treatment of T-AYU-HM Pre at concentrations 50, 100 and 500 µg/ml at 60 min, 90 min, 120 min and 12 h **Table 13**.

TABLE 13: EFFECT OF T-AYU-HM PREMIUM ON % MET-HAEMOGLOBIN OF SICKLE ERYTHROCYTES

Time	% Haemolysis			
	Control	50 µg/ml T-AYU-HM Pre	100 µg/ml T-AYU- HM Pre	500 µg/ml T-AYU- HM Pre
0 Min	13.01 ± 0.99	9.14 ± 1.30	8.79 ± 0.35	5.15 ± 1.86
15 Min	21.39 ± 2.74	14.43 ± 3.16	12.91 ± 4.28	7.04 ± 5.25
30 Min	27.83 ± 6.18	25.54 ± 9.07	19.49 ± 4.85	14.19 ± 1.68
60 Min	36.01 ± 7.50	28.50 ± 6.85	21.54 ± 3.09	12.11 ± 4.92
90 Min	57.36 ± 3.13	48.42 ± 6.20	35.78 ± 7.60	22.98 ± 3.76
120 Min	63.87 ± 1.65	59.54 ± 5.83*	48.49 ± 5.18*	32.22 ± 2.87*
12 Hours	74.37 ± 6.85	66.29 ± 6.57*	55.14 ± 6.52*	32.50 ± 2.46*

n=5, * p<0.05 as compared to control samples using one-way ANOVA followed by Dunnett's Test

Membrane Stabilizing Activity by using Osmotic Fragility:

Membrane stabilizing activity of T-AYU-H: In hypotonic solution (0.55% NaCl solution) % haemolysis for control found to be 50.61 ± 0.73. The % haemolysis of T-AYU-H was found significantly different from that of control. The % haemolysis of T-AYU-H at concentration 50, 100 and 500 µg/ml at 60 min, 90 min, 120 min and 12 h respectively standard Vanilla (10 mM) **Table 10**.

Membrane Stabilizing Activity of T-AYU-HM Premium: In hypotonic solution (0.55% NaCl solution) % haemolysis for control found to be 49.17 ± 0.70. The % haemolysis of T-AYU-HM Pre was found significantly different from that of control. The % haemolysis of T-AYU-HM Pre at concentration 50, 100 and 500 µg/ml at 60 min, 90 min, 120 min and 12 h respectively standard Vanilla (10 mM) **Table 14**.

TABLE 14: EFFECT OF T-AYU-HM PREMIUM ON MEMBRANE STABILIZATION ACTIVITY

Conc. of NaCl (%)	% Met-haemoglobin				
	Control	50 µg/ml T-AYU-HM Pre	100 µg/ml T-AYU-HM Pre	500 µg/ml T-AYU-HM Pre	10 mM Vanillin
0.90	13.71 ± 0.27	13.47 ± 0.39	12.98 ± 0.21	11.81 ± 0.26	12.15 ± 0.25
0.80	14.23 ± 0.26	14.42 ± 0.40	13.57 ± 0.21	12.32 ± 0.28	12.45 ± 0.24
0.70	16.56 ± 0.27	16.69 ± 0.44	15.01 ± 0.21	14.48 ± 0.32	14.72 ± 0.25
0.60	32.06 ± 0.45	28.07 ± 0.68	25.02 ± 0.36	22.25 ± 0.49	23.31 ± 0.36
0.55	49.17 ± 0.70	45.00 ± 1.11	40.07 ± 0.57	34.24 ± 0.75	36.71 ± 0.58
0.50	67.15 ± 0.89	64.13 ± 1.54	59.94 ± 0.87	55.51 ± 1.23	56.83 ± 0.85
0.40	83.16 ± 1.12	82.09 ± 1.98	77.56 ± 1.12	75.66 ± 1.67	75.78 ± 1.14
0.30	90.18 ± 1.22	90.05 ± 2.17	88.16 ± 1.28	86.67 ± 1.92	85.95 ± 1.31
0.10	93.77 ± 1.26	93.20 ± 2.24	90.21 ± 1.31	90.65 ± 2.00	91.04 ± 1.40
0.00	100.00 ± 0.00	96.79 ± 1.37	94.73 ± 1.66	94.47 ± 1.50	

Effect of T-AYU-H on Thrombus Weight in Stasis Induced Venous Thrombosis Model: Increasing doses of T-AYU-H resulted in decrease of thrombus weight. The ASA (30 mg/kg p.o.) dose significantly reduced of thrombus weight and showed 35.33% inhibition of thrombus. Increasing doses of T-AYU-H found to be dose dependently inhibited the thrombus formation. The % inhibition of thrombus formation for T-AYU-H 100, 300 and 500 mg/kg, p.o. was found to be 12.87%, 30.54% and 35.93 % **Table 15**.

TABLE 15: EFFECT OF T-AYU-H ON THROMBUS WEIGHT STASIS INDUCED VENOUS THROMBOSIS MODEL

Groups	Thrombus weight (mg)	Inhibition (%)
Vehicle control (0.5% CMC)	6.68 ± 0.74	0
ASA (30mg/kg p.o.)	4.32 ± 0.57*	35.33*
T-AYU-H (100mg/kg p.o.)	5.82 ± 0.46	12.87
T-AYU-H (300mg/kg p.o.)	4.64 ± 0.43*	30.54*
T-AYU-H (500mg/kg p.o.)	4.28 ± 0.44*	35.93*

n=5, * p<0.05 as compared to control samples using one-way ANOVA followed by Dunnett's Test

Effect of T-AYU-HM Premium on Thrombus Weight in Stasis Induced Venous Thrombosis Model: Increasing doses of T-AYU-HM Premium resulted in decrease of thrombus weight. The ASA (30 mg/kg p.o.) dose significantly reduced of thrombus weight and showed 35.33% inhibition of' thrombus. Increasing doses of T-AYU-HM Premium found to be dose dependently inhibited the thrombus formation. The % inhibition of thrombus formation for T-AYU-HM Premium 100, 300 and 500 mg/kg, p.o. was found to be 15.57%, 33.53% and 36.23 % **Table 16**.

TABLE 16: EFFECT OF T-AYU-HM PREMIUM ON THROMBUS WEIGHT STASIS INDUCED VENOUS THROMBOSIS MODEL

Groups	Thrombus weight (mg)	Inhibition (%)
Vehicle control (0.5% CMC)	6.68 ± 0.74	0
ASA (30mg/kg p.o.)	4.32 ± 0.57*	35.33*
T-AYU-HM Pre (100mg/kg p.o.)	5.64 ± 0.46	15.57
T-AYU-HM Pre (300mg/kg p.o.)	4.44 ± 0.44*	33.53*
T-AYU-HM Pre (500mg/kg p.o.)	4.26 ± 0.82*	36.23*

n=5, * p<0.05 as compared to control samples using one-way ANOVA followed by Dunnett's Test

Reversal of Sickle Erythrocyte: The erythrocyte suspension was deoxygenated with equal volume of 2% w/v sodium metabisulphite ($Na_2S_2O_5$) for 40 min at 37 ºC. Then deoxygenated erythrocyte suspension was treated with PBS (Control) for treatment deoxygenated erythrocyte suspension was treated with 100 µg/ml of test formulations *i.e.* T-AYU-H and T-AYU-HM Premium and for standard, deoxygenated erythrocyte suspension was treated with 10mM Vanillin.

Then, the cells were placed under anaerobic conditions between glass cover slip (Gorecki *et al.*, 1980). Reversal of sickling was observed after 15 min, 30 min and 45 min by using photomicroscope **Table 17** and **Fig. 6**.

TABLE 17: REVERSAL OF SICKLING ERYTHROCYTES

	% of Sickled cells			
Time (Min)	PBS (control)	T-AYU-H (100mg/ml)	T-AYU-HM Pre (100mg/ml)	Vanillin (10 mM)
0	80.14 ± 2.37	80.14 ± 2.37	80.14 ± 2.37	80.14 ± 2.37
15	81.29 ± 3.46	61.21 ± 7.69	54.34 ± 4.64	45.37 ± 2.64
30	80.37 ± 2.14	45.12 ± 3.74	41.25 ± 1.54	34.67 ± 2.39
45	83.74 ± 1.64	19.17 ± 1.69	16.25 ± 3.45	12.45 ± 2.18

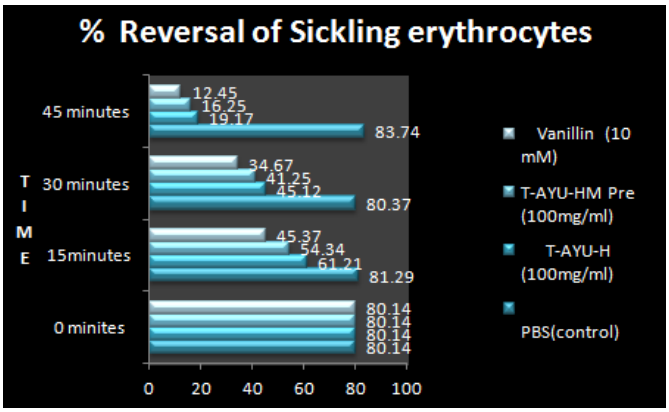


FIG. 6: REVERSAL OF SICKLING ERYTHROCYTES

CONCLUSION: The present work was an attempt to confirm the varsity of claims made by Traditional Ayurvedic Herbomineral formulations for treatment sickle cell anaemia. Pharmacological screening of Herbomineral formulations was carried out by using experimental models for treatment in sickle cell anaemia. Experimental models designed for evaluation of antisickling activity includes sickling induced by hypoxic conditions, membrane stabilization of sickle erythrocytes, t-BOOH induced oxidative stress on sickle erythrocyte and stasis induced venous

thrombosis model. Sickle cell anaemia is a genetic disease due to a mutation in β-chain of haemoglobin. This mutation decreases the affinity of haemoglobin for oxygen. At low oxygen tension, the mutant haemoglobin (HbS) polymerizes inside the red blood cells into a gel or further into Fibers leading to the change of the shape of erythrocytes from their normal globular form into one resembling a sickle [6].

In sickle cell anaemia, pathophysiology is complicated. Local inflammation and thrombosis causes episodic micro vessel occlusion at sites, which induces painful crisis and disability. Free radicals and free iron causes oxidative stress to RBC membrane results in severe hemolysis and microvasculature dysfunction leading to moderate to severe anaemia.

Literature survey revealed that selected herbomineral formulations include various plant extracts and minerals, which can be useful in the treatment of sickle cell anaemia based on their pharmacological action like antisickling, antiplatelet, antithrombotic, anti-inflammatory, analgesic and antioxidant. For instance, Aloe Vera extract which shows potential as antisickling activity, suppresses the oxidative injury and it also showed the antithrombotic activity. *Zingiber officinale* contains essential oil useful in antisickling activity, anti-inflammatory activity which is useful in complications of sickle cell anaemia such as painful crisis. *Punica granatum* contains brevifolin carboxylic acid, brevifolin, 7, 8-Dihydroxycoumarin the constituents having galloyl moiety in their structures and the 7-Ellagic acid which contains the catechol group shows antioxidant and analgesic activities. *Myristica fragrans* shows potent antiplatelet activity, antioxidant activity, *Myristica fragrans* contains the catechol group, (-)-Epicatechain. *Phylanthus emblica*, contains polyphenols like gallic acid and ellagic acid shows antioxidant, anti-inflammatory.

Piper longum shows inhibitory activity on platelet aggregation induced by collagen, arachidonic acid, and platelet- activating factor, *Terminalia chebula* shows antioxidant properties, contains polyphenolic compounds like casuarinin, chebulanin, chebulinic acid or 1,6-di-O-galloyl-β-D-glucose. *Asparagus racemosus* shows antioxidant, cytoprotective activity, Mica Ash useful in anaemia, splenomegaly, aging and general debility, Iron Ash used in treatment of iron deficiency anaemia. These plants and mineral may offer antisickling activity to the formulations.

At low oxygen tension, substituted valine of haemoglobin causes reaction with another haemoglobin molecule, with this haemoglobin polymerization started inside the red blood cells results in a gel or further into fibers leading to the change of the shape of erythrocytes from their normal globular form into one resembling a sickle.

The morphological changes observed in hypoxic conditions showed 60-70% sickled shaped cells in the control(untreated) blood samples, however, when the red blood cells were treated with test formulations showed dose dependent recovery to 27-30% sickled shape cells. Stabilization of membrane in dehydration and inhibition of polymer formation of deoxy HbS may be possible mechanism of action in the sickling of RBC.

Literature survey revealed that the membranes of human erythrocytes from normal haemoglobin blood (HbAA) and sickle haemoglobin blood (HbSS) have varying stabilities after inducing osmotic stress. It has also been suggested that pharmacological agents that alter membrane stability could be applied in the control of sickling process of erythrocytes [8], which is a major physiological manifestation of the sickle cell disease. Decrease in oxygen tension causes polymerization of haemoglobin and further decrease in red blood cell membrane stabilization.

Providing that, in further investigation the role of formulations in erythrocyte membrane stability was determined. The osmotic fragility of cells reflected their withstand in hypotonicity results from its biconcave shape which allows the cell to increase its volume. This osmotic fragility is experimentally denoted by percentage haemolysis at hypotonic saline solution. Haemolysis of sickle RBC decreases with exposure to increasing concentrations of hypotonic saline. The results showed decrease in haemolysis in the test formulations treatment in the hypotonic saline solution. This indicates that test formulations improved the membrane stability of RBC.

Tert-butyl hydroperoxide (t-BOOH) induces oxidative stress in the cell membrane and induces lipid peroxidation in erythrocytes after incubation t-BOOH diffuses freely into the red cell cytosol, where it is reduced *via* free-radical-mediated decomposition by haemoglobin. Reaction of t-BOOH with haemoglobin results in formation of t-butoxyl radicals, which then react with membrane lipids to initiate peroxidation. Haemoglobin gets degraded into methaemoglobin and further oxidation of methaemoglobin by hydroperoxides, results in formation of hemichrome followed by precipitation of hemin, which is crucial to the membrane damage ensuing in haemolysis of RBCs. In the present study sickle cell anemia RBCs incubated with t-BOOH showed increased production of methaemoglobin and hemolysis of cells. The sickle RBC incubated with 0.5mM t-BOOH in presence of the herbal and herbomineral formulations showed increased resistance to hemolysis, dose and time dependent decrease in the formation of methaemoglobin formation was observed, therefore the formulation possess protective effect on t-BOOH induced oxidative stress [2].

Abnormal sickle RBC induce the expression of coagulation mediators, deformed sickle cell membrane results in phosphatidylserine exposure on the cell surface, forms a docking site for hemostatic factors, such as the prothrombinase complex (factor Xa, Va, and II) leading to the activation of the vascular endothelium and stimulate endothelial cells directly by adhesion. The stimulated endothelial cells are poised to recruit rolling and adherent leukocytes in venules by expressing chemokines and cell adhesion molecules such as the selecting and immunoglobulin family members. Activated, firmly adherent neutrophils capture circulating discoid and sickle shaped RBC, leading to transient episodes of thrombus formation and subsequently leads vaso-occlusion crisis that are initiated in the smallest postcapillary venules [6].

Antithrombotic and antiplatelet drugs such as heparin, tinzaparin, aspirin and eptifibatide are under clinical investigation in patients with sickle cell anaemia and vaso-occlusive events showed significant reduction in the number of days with severe pain scores, overall duration of painful crisis and duration of hospitalization. Test formulations effectively inhibited thrombus formation as compared to control and effective as aspirin in high dose group, which showed significant thrombus weight reduction. Thrombus inhibitory action of test formulations could be one of the important parameters in reducing the painful crisis and haemolytic crisis. Vaso-occlusion is the major pathophysiological consequences for the blockage of microvasculature. Vaso-occlusion induced due to stasis involves blockage of blood flow in blood vessels in various body parts. Such vaso-occlusive crisis worsens as the disease progresses.

The investigation provided experimental evidence indicating potential application of the formulations T-AYU-H and T-AYU-HM premium effective in the treatment of sickle cell anaemia. The ability of the formulations, in this study, to normalize the sickle erythrocytes represents a rational explanation for the use in treating sickle cell anaemia; there is further scope to evaluate formulations on solubilization of deoxyhaemoglobin S and oxygen carrying capacity. The formulations have significantly decreased the haemolysis of sickle RBCs by reducing the oxidation of haemoglobin. All two formulations showed *in-vitro* membrane stabilization activity in the presence of osmotic stress, there is further scope for studies on Gardos channels and membrane bound enzymes (Na^+, K^+, Ca^{2+} ATPase and Mg^{2+} ATPase). *In-vivo* stasis induced venous thrombosis model in SD rats after oral administration of formulations produced a dose dependent decrease in thrombus weight, signifies anti-thrombotic activity of test formulations, specific inhibitory activity of formulations is needed to evaluated. The present investigation provides evidence to support the claims of the Ayurvedic herbomineral formulations T-AYU-H and T-AYU-HM premium as antisickling formulations.

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