Inhibition of erythrocyte sickling *in vitro* by aqueous extracts of *sphaeranthus indicus* flowers and *Ziziphus jujuba* root

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Abstract
Sickle cell disease (SCD) results from a mutation in the hemoglobin inside the red blood cells, where a glutamic acid at position 6 is replaced by a valine. Many phytomedicines have been identified as potential antisickling agents, stemming from reported usage as ethnomedicines by the local folk. In present study, aqueous extracts of *Sphaeranthus indicus* Flowers and *Ziziphus jujuba* Root for their possible antisickling activity was evaluated by spectrophotometrically. Both extracts caused significant (p<0.001) reduction in HbS polymerization. Aqueous extract of *S. indicus* (250 and 500µg/ml) at 120 sec showed maximum reduction in polymerization, 52.03 ± 0.14 and 62.40 ± 0.19% respectively. However, 1000 µg/ml aqueous extract of *S. indicus* at 90 sec exhibits highest reduction in polymerization, 65.83 ± 0.12. Aqueous extract of *Z. jujuba* (250 and 1000µg/ml) at 120 sec showed maximum reduction in polymerization, 61.53±0.20 and 72.90 ± 0.21% and 500 µg/ml aqueous extract of *Z. jujuba* showed maximum reduction 67.77 ± 0.22% at 150 sec. We conclude that aqueous extracts of *Sphaeranthus indicus* Flowers and *Ziziphus jujuba* Root are potential candidate for therapy and management of sickle cell disease.

Keywords: *Sphaeranthus indicus*, *Ziziphus jujuba*, Antisickling activity.

1. Introduction
Hyperlipidemia every year approximately 100,000 children in the world are born with sickle cell disease (SCD) which is a genetic disorder. This disease is considered as a public health problem in many countries, but with a major burden in Africa [1]. SCD also known as sickle cell anemia or drepanocytosis, is an inherited illness which is caused by an abnormal hemoglobin. The SCD causal hemoglobin (Sickle hemoglobin or S hemoglobin, HbS), comes from a mutation at the 6th position of the beta globin chain, which led to the substitution of glutamic acid, a polar amino acid, by valine a non-polar amino acid. This structural modification influences significantly physical and chemical properties of hemoglobin, hemoprotein that are responsible for the transport of oxygen from the lungs to other tissues in the body [2, 3]. This mutation decreases the affinity of hemoglobin for oxygen. At low oxygen tension, the mutant hemoglobin polymerizes inside the red blood cell into a gel or further into fibers leading to a drastic decrease in the red cell deformability. Polymerization and precipitation of S hemoglobin within the erythrocytes cause the change of the shape of erythrocytes from their normal globular form into one resembling a sickle. Sickling of blood cells is the cause of precocious hemolysis of erythrocytes and various complications of SS subjects [2, 3].

The first-line clinical management of SCD includes medullar transplantation, repeated blood transfusion to stabilize the patient's hemoglobin level. Despite the fact that the molecular biology of sickle cell is well characterized, there is at present no specific drug that can prevent or permanently cure the disease [4]. The lack of effective therapy for sickle cell anaemia has prompted investigation into a large number of antisickling agents [5-7]. Some of the antisickling agents investigated exhibits varying degrees of toxicity, and/or cause haemolysis of the sickle red blood cell at the effective dose levels and are therefore unsuitable for clinical use and also quite expensive. Therefore, there is a need for more definite and effective treatments for the disease. Herbal extracts have been used in Indian folk medicine for decade in the management of various ailments.

Such two medicinal plants, *Sphaeranthus indicus* Linn. And *Ziziphus jujuba* Mill. Were selected for present study. The genus *Sphaeranthus* belongs to Family Astraceae, this ornamental herb commonly known as Gorakhmundi. All the parts of the plants have medicinal uses. In folk medicine, the plant is reportedly used in treating epileptic convulsions, mental illnesses and hemicranias [8]. The whole herb is used in ayurvedic preparations to treat epilepsy and mental disorders [9]. It is used to treat vitiated conditions of hemicranias, jaundice, hepatopathy, diabetes, leprosy, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia and skin diseases. It is also used as anervine tonic. (Rhamnaceae), a spiny deciduous shrub or small tree. *Ziziphus jujuba* is being used by tribal Adivasies in eastern parts of Jalgaon District (Maharashtra State) influencing injuries small cuts and or animals bite, attack and wounds [10]. Various activities like anti-inflammatory [11]; sedative and hypnotic [12]; anticancer, antiretroviral [13]; anti-complementary [14] and antioxidant [15] has been reported.

The present study was performed with the aim of evaluating the antisickling activity of aqueous extracts of flowers of *Sphaeranthus indicus* Linn. And roots of *Ziziphus jujuba* Mill. For the best of our knowledge, these plants have not yet been scientifically investigated for its antisickling properties.

2. Materials and Methods
2.1 Collection of plant specimen
The plants were collected from North Maharashtra Region in the period of September 2016. The plant was identified by Dr. Maroti Deshattiwar, Department of Botany, Moolji Jaitha College, Jalgaon.

2.2 Preparation of extract of plant specimen
Plant parts were separated, washed under continuous current of distilled water for 15 min and dried. After complete drying

the materials were crushed and grinded to form coarse powder. Powdered plant materials were exhaustively extracted in Soxhlet apparatus with distilled water. The extracts so obtained were then filtered to remove any suspended impurities. Each extract was concentrated under reduced pressure and controlled temperature (55^0C to 60^0C). Obtained extracts were labeled as Aq Si and Aq Zj for *S. indicus* and *Z. Jujuba* respectively and then preserved in dry, cool condition in desiccator and used for experimental purpose.

2.3 Collection of blood sample

The blood samples used in the evaluation of the antisickling activity of the plant extracts in this study were taken from patients known to have sickle cell disease. The blood samples were collected in EDTA tubes and stored for maximum a few hours for the experiment.

2.4 Washing and Preparation of erythrocytes haemolysate

The erythrocytes were washed by centrifugation method as described by Alabdallat [16]. Within 2 h of collection of blood samples, portion of 1.0 ml of the samples were introduced into centrifuge test tubes containing 3 ml of sterile normal saline. The erythrocytes were separated from plasma by centrifugation at 1200 X g for 10 min, washed three times by the same centrifugation method with sterile normal saline. The erythrocytes were finally re-suspended in 1.0 ml of this buffer and stored at 4^0C. The washed erythrocytes were lysed by freezing/thawing [17, 18]. The erythrocytes haemolysate was used for polymerization analysis.

2.5 Polymerization study

Sodium metabisulphite ($Na_2S_2O_5$) induced polymerization of HbS molecules was ascertained [19]. The underlying principle is that HbS molecules undergo gelation when deprived of oxygen; sodium metabisulphite was used as reductant. The level of polymerization was monitored by recording increasing absorbance of the assay mixture with time. A 0.1 ml of HbS hemolysate was introduced into a test tube, followed by 0.5 ml of 150 mM phosphate buffered saline solution (NaCl 150mM, 120mM $Na_2HPO_4.2H_2O$, 30mM $NaH_2PO_4.2H_2O$ and pH– 7.4) and one ml of distilled water. The mixture was transfer into cuvette and 3.4 ml of 2% aqueous solution of sodium metabisulphite was added. The absorbance of the assay mixture was recorded with a spectrophotometer at every 30 second for 180 second at λmax = 700 nm (control sample). This procedure was repeated by substituting the distilled water with 1.0 ml of corresponding three increasing concentrations (250, 500 and 1000 µg/ml) of the both extracts (test sample) and parahydroxy benzoic acid (5mg/ml).

Percentage polymerization = $[A_{t/c}/Ac_{180^{th}sec}]$ X 100

Where $A_{t/c}$ = Absorbance of test/ control sample at time t sec.

$Ac_{180^{th}sec}$ = Absorbance of control sample at the 180^{th}sec.

2.6 Statistical analyses

All data were expressed as mean ± SE and the ANOVA was applied to determine the significance of the difference between the standard group and experimental groups by Dunnett's test.

Table 1: Percentage reduction of HbS polymerization in presence of aqueous extracts of flowers of *S. indicus* and roots of *Z. Jujuba* with time. Each value expressed as mean ± SE, n=6, a = P<0.001, b = P<0.01 Vs Standard

Time(sec) / Groups	0	30	60	90	120	150	180
Standard	51.51±0.17	51.83±0.21	52.21±0.12	52. 34±0.19	53.68±0.17	53.21±0.14	53.58±0.24
Aq Si 250	49.90±0.14	50.19±0.18	50.26±0.20	50.37±0.23	52.03±0.14	51.33±0.22	51.09±0.11
Aq Si 500	60.83±0.15	61.08±0.24	61.39±0.15	61.79±0.16 [a]	62.40±0.19[a]	62.28±0.15 [a]	62.17±0.19 [a]
Aq Si 1000	63.40±0.17 [a]	63.43±0.15 [a]	64.12±0.13	65.83±0.12 [a]	65.42±0.20 [a]	64.12±0.15 [a]	64.68±0.21 [a]
Aq Zj 250	57.04±0.14 [b]	57.78±0.17 [b]	58.21±0.23 [b]	59.11±0.20 [b]	61.53±0.20 [a]	60.41±0.18 [a]	59.55±0.13 [b]
Aq Zj 500	65.24±0.12 [a]	65.49±0.20 [a]	65.65±0.21 [a]	65.98±0.22 [a]	67.25±0.18 [a]	67.77±0.22 [a]	66.21±0.18 [a]
Aq Zj 1000	73.17±0.20 [a]	74.00±0.15 [a]	74.16±0.10 [a]	74.42±0.18 [a]	72.90±0.21 [a]	72.85±0.13 [a]	72.30±0.15 [a]

3. Results and Discussion

The results presented in Table 1 showed that aqueous extracts of both plants caused significant (p<0.001) reduction in HbS polymerization when compared to standard groups. Aqueous extract of *S. indicus* (250 and 500µg/ml) at 120 sec showed maximum reduction in polymerization, 52.03 ± 0.14 and 62.40 ± 0.19% respectively. However, 1000 µg/ml aqueous extract of *S. indicus* at 90 sec exhibits highest reduction in polymerization, 65.83 ± 0.12. Aqueous extract of *Z. jujuba* (250 and 1000µg/ml) at 120 sec showed maximum reduction in polymerization, 61.53 ± 0.20 and 72.90 ± 0.21% and 500µg/ml aquesous extract of *Z. jujuba* showed maximum reduction 67.77 ± 0.22% at 150 sec. When we compared the all test groups of both plants, aqueous extract of *Z. jujuba* at 1000µg/ml caused highest reduction, 74.42 ± 0.18% in Hb polymerization at 90 sec, this fell gradually so that at 180 sec, it was 72.30 ± 0.15%.

Addition of 250, 500 and 1000 µg/ml aqueous extracts of *S. indicus* and *Z. jujuba* to the assay mixture caused reduction of deoxy HbS polymerization within the experimental period (t = 0 to 180 sec). However, the capacity of three mentioned concentrations of *S. indicus* and *Z. jujuba* to inhibit polymerization of deoxy HbS molecule diminished between 90 to 180 sec experimental time.

In vitro deoxygenation of haemolysate HbS molecules by sodium metabisulphite caused aggregation and polymerization of the individual haemoglobin molecules. This process of gelation (polymerization) of haemoglobin molecules resulted in increasing absorbance of the assay solution.

Previous reports have proposed the use of herbal preparations as candidate for management of sickle cell disease [20-25]. Their proposals were drawn from the fact that these plant extracts, from *in vitro* studies, exhibited anti-sickling/polymeriztion property. The findings of this research are comparable to those previous reports.

Research findings have established that the capability of a biomolecule to impede *in vitro* polymerization depends on one or combinations of the following options: (a) the tendency and

efficiency to bind to the complimentary contact region/site of deoxy HbS monomers [26-28]; (b) modification of amino acid residues that contribute to the three dimensional structures of HbS contact region and other critical sites [29-31]; (c) stabilization of the R (relaxed) state of HbS molecule [29, 30, 32, 33]. The diminishing capacity of the plant extracts to inhibit polymerization of deoxy HbS molecule with progression of experimental time suggest that the constituents of the extracts did not covalently modify the amino acid residues unlike other reported compounds [26, 27, 28,30, 31].

Rather, the anti-polymerization principles of the plant extracts may have formed a relatively weaker hydrophobic interaction with the contact regions of HbS molecules that temporarily reduced polymerization of HbS monomers. Furthermore, the protein/ligand associations may have transiently stabilized the R-state conformation, but were subsequently displaced by more thermodynamically favorable interactions that cause and promoted haemoglobin polymerization [34]. Therefore, the capacity of the two extracts to inhibit HbS polymerization was not sustained with the progress of experimental time.

4. Conclusion

For the first time we recorded this results. The results obtained in this study have shown significant *in vitro* antisickling activity of aqueous extracts of flowers of *S. indicus* and roots of *Z. jujuba*. Further study on its isolation and characterization of active principle for antisickling activity is warranted.

5. References

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