



An *in vitro* study on anti-sickling activity of *Terminalia arjuna* and *Terminalia bellirica*

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ABSTRACT

Background & Aim: Combretaceae family is known for efficacy in the management of sickle cell disorder (SCD). Based on the earlier reports of anti-sickling property of *Terminalia* genus the study investigates *in vitro* antisickling properties of *Terminalia arjuna* and *Terminalia bellirica*.

Experimental: Anti-sickling assay of methanolic and aqueous extract of *T. arjuna* and *T. bellirica* on sickled RBCs were evaluated at different concentrations. The osmotic fragility test was done to explore the effect of extracts on haemoglobin S solubility and sickle cell membrane stability. Additionally antioxidant activity and phytochemical analysis of extracts was evaluated.

Results: Treatment with the plant extracts at different concentrations showed decrease in percentage of sickled cells. Extract of *T. arjuna* had a significantly higher ($p < 0.05$) antisickling activity at the tested concentrations of 10 mg/ml. The methanolic extract at 10 mg/ml showed the best cell membrane stability as compared to *T. bellirica* extract. It was observed that, *T. bellirica* extract was deficient of alkaloid and tannins. Moreover, all extracts/fractions showed antioxidant activity, although lesser as compared to the standard.

Recommended applications/industries: It was observed that the leaf extracts of *T. arjuna* has a significant antisickling effect, hence it can be potentially used in clinical management of SCD. The bioactive compounds can be used to supplement as well as management drug for sickle cell disease.

1. Introduction

About five million people yearly in the world are affected by Sickle cell disease (SCD) and approximately 1,00,000 children in the world are born each year with SCD. It is considered as a public health problem, particularly in west and central Africa and Indian subcontinent. (Moody et al., 2003, Mpiana et al., 2007,

Mandot et al., 2009). Sickle cell anemia is an autosomal genetic blood disorder where concave blood cells are changed to abnormal sickle shaped red blood cells (RBCs). In sickle hemoglobin, glutamic acid is replaced by valine in position 6 (Mpiana et al., 2013) causes a reduction in the solubility of sickle cell hemoglobin (HbS) and its polymerization to form a mass of fibres which changes shape of erythrocytes into a sickle shape (Clarke and Pazdernik, 2013). The therapies include

bone marrow transplantation, gene therapy along with frequent blood transfusions, use of hydroxyurea, but are expensive (Mpiana et al., 2011, Mpiana et al., 2013, Makani et al., 2013).

The mechanism of antisickling agents includes inhibiting polymerisation of haemoglobin or modifying membrane stability and red cell hydration evaluated by measuring RBC haemolysis (Mpiana et al., 2010). The *Terminalia* genus belongs to the Combretaceae family with 200 species (Pietrovski et al., 2006). *T. arjuna* and *T. bellirica* are Asian species extensively used Pietrovski in traditional medicine. *T. arjuna* used as analgesic, antiinflammatory, antioxidant, cardiac stimulant whereas, *T. bellirica* as expectorant, laxative, in anaemia, and anthelmintic properties (Cock, 2015).

The therapeutic activities of traditional practice medicine are mostly unidentified. There is a need to find out the remedies which are more economical and easily available. This study is a step forward to find out a potent product from the plants which can reduce the suffering of SCD people. This work investigates the antisickling, antioxidant and erythrocyte membrane stabilizing activities of *T. arjuna* and *T. bellirica* leaf extracts which were yet not been carried out in the knowledge.

2. Materials and Methods

2.1. Plant material and preparation

The plants selected were used for the treatment of anemia based on the guidance of traditional herbal healers and confirmed from Ethno-medicinal plants used by tribal's in central India (Gangarde et al., 2003). Leaves of *T. arjuna* (Roxb.) Wight & Arn. and *T. bellirica* (Gaertn.) Roxb. were collected from the central India and identified from the Herbarium of the Department of Botany, Govt M.V.M., Bhopal and providing them specimen no.: Bot/08/2015. The dried leaves were pulverized, labeled and stored in airtight containers at 20°C.

2.2. Phytochemical tests

The alkaloids, anthraquinones, cardiac glycosides, flavonoids, saponins and tannins present in the powdered leaf were analyzed using established protocols (Adesanya and Sofowora, 1983 Harbone, 1998).

2.3. Extraction

Cold extraction was conducted with powdered leaves (500 g) with 2 L of methanol in amber bottles for 48 hours, with intermittent shaking. The crude methanol extract was filtered (0.45µm Whatman filter) and concentrated using a rotary evaporator. In another set, powdered leaves were soaked in sterile; distilled water for three days at 27°C and the aqueous extract was collected by filtration and concentrated in a rotary evaporator. 0.9% NaCl was used to dilute both extracts to give 10, 5, 1mg/ml concentrations for subsequent antisickling assay.

2.4. Biological material

The blood samples were taken from anemic adolescent patients attending the People's Hospital and Research Centre, Bhopal. None of the patients had been transfused recently with HbAA blood. A written, informed, witnessed consent was signed by the patients who donated blood for the study. The research project had the approval with reference no. PU/CSR/SI/30, from Centre for Scientific Research and Development, People's University, Bhopal, India. Blood was collected in sodium EDTA bottles with gentle mixing. All experiments were performed with fresh blood. In order to confirm their sickle cell nature, the blood samples were first characterized by D-10 HPLC (Bio Rad). Packed erythrocytes were collected by washing in normal saline and were then stored at 4°C (Egunyomi et al., 2009).

2.5. Membrane stability of erythrocytes

The osmotic fragility of erythrocytes was determined by placing the cells in a graded series (0.00-0.85% with a difference of 0.10%) of buffered saline, pH 7.4 in 1 ml of each extract (10 mg/mL) to which 50 µl sickle blood was added. The mixture was incubated at 25°C for 24 h, and after incubation the mixture was centrifuged at 3000 rpm for 15 min and optical density of the supernatant was recorded at 540 nm against blank made of 0.85% buffered saline (Jaja et al., 2000). A plot of lysis (%) versus NaCl concentration was obtained to determine the concentration of saline causing 50% haemolysis of the RBC (mean corpuscular fragility).

2.6. Antisickling activity

In vitro anti-sickling activity

Bioassay of crude methanol and aqueous extract of *T. arjuna* and *T. bellirica* were carried out, viz. inhibition of sickling (antisickling) and reversal of

sickled erythrocytes analyzes. The antisickling activity was carried out by following the modified protocol of (Sofowora et al., 1979). The washed erythrocytes and plant extracts were mixed at equal concentration (0.5 ml) in an uncovered test tube. Methanolic and aqueous sample extracts of different concentration were taken and incubated at 37°C for 3 h with occasional shaking. To the mixture, five drops of 2% sodium metabisulphite were added and mixed thoroughly. The test tubes were sealed with liquid paraffin and incubated at 37°C for 30 min. Samples were then taken in duplicate from the different mixtures at 0 min after which the systems were incubated at an interval of 30 min, four times. Method of (Egunyomi et al., 2009) was used to prepare smear and count sickled and unsickled cells. The positive and negative control was p-hydroxybenzoic acid (5 mg/ml) and normal saline respectively. To determine the percentage of sickling inhibition was calculated using the formula of Moody et al. (2003).

In sickling reversal activity of crude extracts, washed erythrocytes were equally (0.5 ml each) mixed with freshly prepared 2% sodium metabisulphite in a clean test tube and incubated at 37°C for 30 min and observed under the microscope (Oduola et al., 2006). To the blood-metabisulphite mixture equal volume of extracts were added and incubated at 37°C for another 30 min. Samples were observed at 0 min and at 30 min interval for up to 2 h. The smear preparation, counting of sickled and unsickled cells and microscopic images of degranocytes were examined (Egunyomi et al., 2009).

2.7. Antioxidant activity

Scavenging Activity

The scavenging activity of the two methanolic and aqueous extracts on DPPH (1,1-diphenyl-2-picrylhydrazyl) was determined using the method of Choi et al. (2002) with minor modifications. The changes in color were measured spectrometrically at 518 nm. In 3 mL of each diluted extract, 1 ml of 0.1 mM DPPH methanol solution was added. All the extracts were tested at final concentrations ranging from 10, 5 and 1 mg/mL. At different concentrations of extract with a final concentration of 3 mL each, one milliliter of 0.3 mM DPPH ethanol solution was added to give test solutions, while 1 mL of ethanol was added to 3 mL of sample to produce the blank solutions. The solutions were allowed to react at room temperature for 30 min in the dark. The absorbance were measured at 518 nm and converted into percentage antioxidant activity using the

equation: The negative control was 1 mL of DPPH solution in 3 mL of absolute ethanol.

$$\% \text{ Inhibition} = [(AB - AA)/AB] \times 100,$$

Where AB: absorption of blank sample; AA: absorption of tested samples.

Moreover the IC₅₀ value and the kinetics of DPPH activity were determined to know the efficacy of the extract. The positive controls were Ascorbic acid and butylated hydroxytoluene (BHT).

2.8. Statistical analysis

All the experiments were repeated twice and the mean data was used for interpretation. The probability of the finding and its significance of differences were analysed.

3. Results and discussion

Anise Out of fifty blood sample collected, sickle cell disease was identified in twelve using D 10 HPLC (Fig. 1).

3.1. Phytochemical screening

The qualitative phytochemical screening of plant extracts provides necessary information regarding chemical constituents. The results of phytochemical screening of leaves have been shown in Table 1. The *T. arjuna* showed the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, saponins, tannins while alkaloids and flavonoids were absent in *T. bellirica*. Alkaloids were used as nerve stimulant, muscle relaxant (Robert and Wink, 1998). Alkaloid may be useful in alleviating symptoms of pains. Anthroquinone increases peristaltic action. Flavonoids possess potential pharmacological activities such as antioxidant activity, vitamin C sparing activity (Middleton and Kandaswami, 1992). Antisickling activity of may be due to presence of antioxidant activity. The presence of cardiac glycosides may be potent in curing cardiac insufficiency, cough and circulatory problems. Tannins are phenolic derivatives, may be useful in cleansing the surface of the skin ulcers that develop as a result of sickle cell disease (Kenner and Yves, 1996).

3.2. Antisickling activity

Tables 2 and 3 are the observation of antisickling activities of both methanol and aqueous extracts of *T.*

arjuna and *T. bellirica* whereas morphological changes in degranocytes showed in Fig. 2. At the tested concentrations of 5 and 10 mg/ml, both the methanol and aqueous extracts of *T. arjuna* exhibited antisickling activity as compared with *p* hydroxybenzoic acid (PHBA) (positive control). The exhibited antisickling activity was concentration dependent. The antisickling activity exhibited by the aqueous extract of *T. arjuna* was significantly higher ($p < 0.05$) at the three tested concentrations of 1, 5 and 10 mg/ml as compared to its methanol extract. In a trend that contrasts the antisickling activity of *T. arjuna*, *T. bellirica* had a weak antisickling activity. Methanolic and aqueous extract at 10 mg/ml exhibited significantly lower ($p < 0.05$) antisickling activity than positive control.

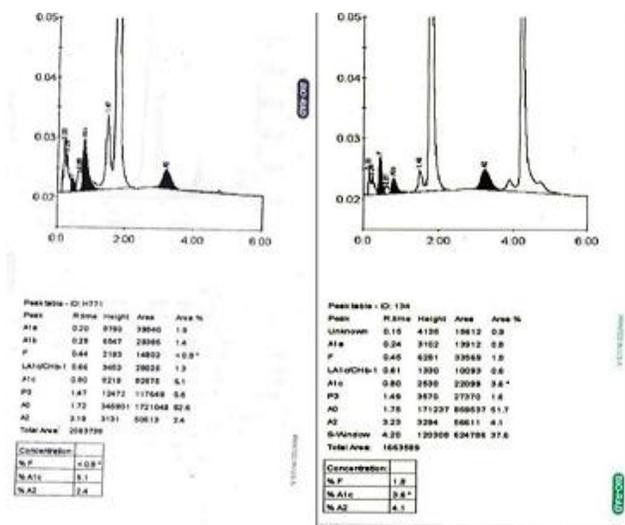


Fig. 1. Chromatogram of blood sample; A: Normal haemoglobin, B: Haemoglobin S.

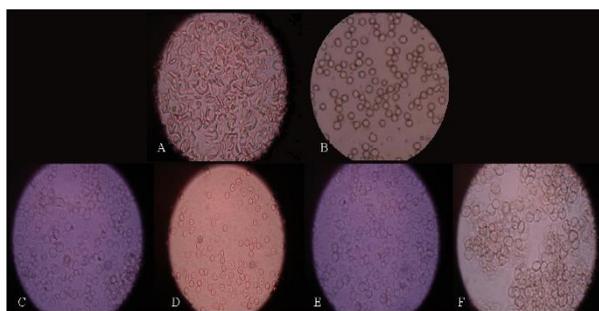


Fig. 2. Morphology of degranocytes; Sick cell blood (A), Treatment with positive control (PHBA) (B), Treatment with methanolic extract of *T. arjuna* (C), Treatment with aqueous extract of *T. arjuna* (D),

Treatment with methanolic extract of *T. bellirica* (E), Treatment with aqueous extract of *T. bellirica* (F), X500.

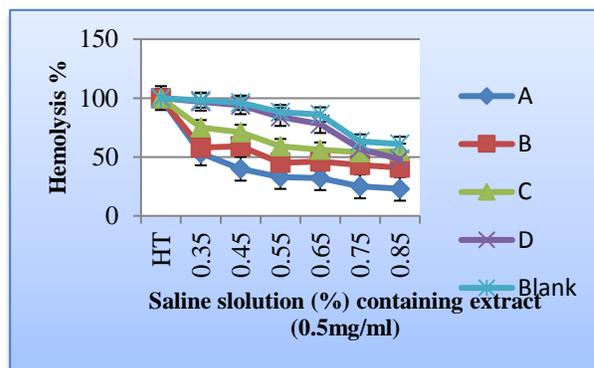


Fig. 3. Percentage of hemolysis of Sickling cells after treatment with 0.5 mg/ml of extract. (A): Aq. extract *T. arjuna* leaves; (B): Methanolic extract *T. arjuna* leaves; (C): Aq. extract *T. bellirica* leaves (D): Methanolic extract *T. bellirica* leaves; HT: Total hemolysis.

Table 1. Phytochemical screening of leaves of *T. bellirica* and *T. arjuna*.

Secondary metabolite	<i>T. arjuna</i>	<i>T. bellirica</i>
Alkaloids	+	-
Anthroquinones	+	+
Cardiac glycosides	+	+
Flavonoids	+	-
Saponin	+	+
Tannins	+	+

The result of sickling reversal bioassay shown in tables 2 and 3 revealed a trend similar to that of the presently reported antisickling activities of both *T. arjuna* and *T. bellirica*. The sickling reversal activities of the investigated plant were concentration dependent. The solvent used in extraction of *T. arjuna* had no significant effect ($p > 0.05$) on sickling reversal activity.

Antisickling activities of leaf of *T. arjuna* and *T. bellirica* verify their use in tribal medicine for SCD management. The antisickling activities given by positive control were maintained throughout incubation period, while of plant extracts activities was decreased after 1 h. It may take into consideration that lower antisickling activity of *T. bellirica* than *T. arjuna* may be due to the absence of alkaloids and flavonoids. This confirms that all component of therapeutic mixture are necessary for activity (Gillete et al., 2004).

3.3. Erythrocyte membrane stability activity

Osmotic fragility of erythrocytes indicates the integrity of red cell membranes in pathological and normal states (Rai et al., 2009). Result of the erythrocyte osmotic fragility showed a significant decrease of the percentage of hemolysis while increasing the concentration of salt solution (Fig. 3). All the extracts tested showed a lower hemolysis percentage compared the control. Among the four fractions tested, aqueous fraction of *T. arjuna* presented the most significant reduction of hemolysis compared to methanolic fraction of same plant and aqueous and methanolic fraction of *T. bellirica* plant. Hence, it could have good guarding effect of the erythrocyte membrane.

3.4. Antioxidant activity

In order to determine *in vitro* antioxidative activity of antioxidant compounds, DPPH radical is widely used. The reduction of DPPH into stable radical indicated by color change measured at 518 nm wavelength. The DPPH scavenging capacity of aqueous leaf fraction of *T. arjuna* (10.8 µg/mL) was the highest among the extracts tested, while methanolic leaves fraction of *T. arjuna* (12.3 µg/mL) had slightly less DPPH scavenging capacity (Table 4). Though, IC₅₀ value of all the extracts

recorded lowest value, methanolic leaf fraction of *T. bellirica* (16.2 µg/mL) demonstrated better scavenging activity than aqueous leaf fraction *T. bellirica* (20.6 µg/mL). Among the positive controls, ascorbic acid (1.6 µg/mL) was found to have better DPPH radical scavenging ability than BHT (1.7 µg/mL). Flavonoids are good source of antioxidant constituents of plants and along with possess radical-scavenging or chelating activities (Miliauskas et al., 2004).

4. Conclusion

This study evaluated the phytochemical screening and the *in vitro* antisickling, erythrocyte membrane stability activity and antioxidant activity of leaf extract of *T. arjuna* and *T. bellirica*. These plants displayed promising *in vitro* antisickling and antioxidant effects. The ability of extract to show such pharmacological properties may give justification for the use of *Terminalia* plant by the traditional folklore to treat SCD. This has not been previously reported as an antisickling plant in the traditional medicine. Therefore further studies are indicated to study active compound and the toxicity profiles in transgenic mice models.

Table 2. Antisickling effect (% inhibition of sickling) of methanol and aqueous extract of *T. arjuna* leaves

Time of incubation (min)	Normal saline	p-Hydroxy benzoic acid (PHBA)	Methanol extract (mg/ml)			Aqueous extract (mg/ml)		
			10	5	1	10	5	1
0	- (0)	0 (0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
30	- (2)	46 (53)	61(43)	53(38)	21(35)	64(45)	60(40)	23(34)
60	(4) -	49 (57)	69(46)	60(45)	30(38)	73(50)	64(49)	35(40)
90	- (5)	54 (63)	60(52)	58(49)	23(41)	63(54)	61(53)	27(49)
120	- (8)	57 (66)	47(59)	32(53)	12(49)	50(60)	39(57)	20(55)

Values in parenthesis are percentage sickling reversal of 2% metabisulphite induced sickled cells. All values are means of triplicate determinations.

Table 3. Antisickling effect (% inhibition of sickling) of methanol and aqueous extract of leaves of *T. bellirica*

Time of incubation (min)	Normal saline	p-Hydroxy benzoic acid (PHBA)	Methanol extract (mg/ml)			Aqueous extract (mg/ml)		
			10	5	1	10	5	1
0	- (0)	0 (0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
30	- (2)	46 (53)	32(22)	19(11)	9(5)	18(16)	12(5)	5(0)
60	(4) -	49 (57)	47(24)	35(14)	13(5)	38(18)	16(6)	6(1)
90	- (5)	54 (63)	31(27)	22(18)	9(7)	22(22)	11(8)	4(1)

120 - (8) 57 (66) 15(30) 15(20) 8(8) 15(22) 9(9) 2(2)

Values in parenthesis are percentage sickling reversal of 2% metabisulphite induced sickled cells. All values are means of triplicate determinations.

Table 4. Antioxidant activities of methanolic and aqueous leaf extracts of *T. arjuna* and *T. bellirica* in DPPH assay.

Samples	DPPH IC ₅₀ , µg/mL
Aqueous extract of <i>T. arjuna</i>	10.8±0.47
Methanolic extract of <i>T. arjuna</i>	12.3±0.37
Aqueous extract of <i>T. bellirica</i>	20.6±0.64
Methanolic extract of <i>T. bellirica</i>	16.2±0.55
Ascorbic acid	1.6±0.14
BHT	1.7±0.07

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