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Hydroalcoholic Extract of *Alstonia scholaris* Arrests Invasion of *Plasmodium falciparum* by Effective RBC Membrane Stabilization

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LBG and HH designed the study, performed the statistical analysis, wrote the protocol and author DJ wrote the first draft of the manuscript. Authors DJ, SG and UJ managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Traditionally medicinal plants play a key role in malaria control. Several studies have demonstrated that alkaloids have excellent antiplasmodial potential. *Alstonia scholaris* is rich in alkaloid content; despite this fact its antimalarial potential has been less explored. Therefore, the main objective of our study was evaluation of the possible anti-plasmodial efficacy of hydro-alcoholic extract of *Alstonia scholaris* leaves, with specific emphasis on its role in stabilization of the RBC membrane.
Place and Duration of Study: Department of Zoology, BMT and Human Genetics, Gujarat University, Gujarat, India, between December-2014 to May- 2016.
Methodology: In order to assess its acclaimed potentials, a hydro-alcoholic extract was prepared

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from leaves of *Alstonia scholaris*, and tested for its antiplasmodial activity, using specific assays for *in vitro* percentage inhibition of entry of parasites and erythrocyte membrane stabilization. In addition, anti-oxidant activity was estimated using DPPH assay.
Results: It was observed that the plant extract had significant antiplasmodial activity. The EC_{50} value of MRC 2 was 8.87 µg/ml and 34.27 µg/ml on RKL 9 with very good antioxidant activity.
Conclusion: This plant could be an effective antimalarial agent which could be used alone or in combination, to effectively control this dreaded parasite protozoan.

Keywords: Alstonia scholaris hydro-alcoholic extract; antioxidant activity; erythrocyte membrane stabilization; antiplasmodial activity.

1. INTRODUCTION

Due to the increasing drug resistance of malarial parasites, malaria remains one of the life-threatening diseases of the developing world. In 2015, approximately 3.2 billion people – nearly half of the world's population – were at risk of malaria [1]. The development of resistance to drugs poses one of the greatest threats to malaria control and results in increased malaria morbidity and mortality. Resistance to currently available antimalarial drugs has been confirmed in only two of the four human malaria parasite species, *Plasmodium falciparum* and *P. vivax*. It is still unknown if *P. malariae* or *P. ovale* have developed resistance to any antimalarial drugs.

Chloroquine resistance has rampantly spread to nearly all corners of the world where *falciparum* malaria is transmitted. *P. falciparum* has also developed resistance to nearly all of the other currently available antimalarial drugs, such as sulfadoxine/ pyrimethamine, mefloquine, halofantrine and quinine. Although resistance to these drugs tends to be much less widespread geographically, in some areas of the world, the impact of multi-drug resistant malaria can be extensive. Most recently, a low-grade resistance to artemisinin-based drugs has emerged in parts of Southeast Asia [2].

Herbal drugs constitute a major share of all the officially recognized systems of health in India *viz*. Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy. More than 70% of India's 1.1 billion population still use these non-allopathic systems of medicine [3]. Dependence on plants is primarily due to their safety, effectiveness, cultural preferences, inexpensive-ness and abundant availability all the time. Despite the recent successes in rational drug design and synthetic chemistry techniques by pharmaceutical companies, natural products and particularly medicinal plants have remained an important source of new drugs [4,5].

Alstonia scholaris belongs to family Apocynaceae that grows throughout in India. This plant is evergreen and a common medicinal plant of India, the Asia- Pacific, Southern China and Queensland [6]. It is useful in fever, diarrhoea, dysentery, skin diseases, ulcers, asthma, bronchitis, cardiopathy, agalactia and debility. Nadkarni et al. [7] have shown that the milky exudate is bitter and is good for ulcers. Various bark extracts of *Alstonia scholaris* have been proven to have effective anticancer, antifertility, immunomodulatory and wound healing activities. In addition, the leaf extracts have been used for antimicrobial, analgesic and antiinflammatory purposes [8].

Several studies have demonstrated that several phytochemicals viz. alkaloids, flavonoids, terpenoids, glycosides have excellent antiplasmodial potential [9]. *Alstonia scholaris* is rich in alkaloid content; however, despite this fact its antimalarial potential is less explored. There are only two reports, which confirmed the anti-malarial potential to *Alstonia scholaris* [10,11]. Khyade et al. [12] have reviewed the potential significance of the extract of *Alstonia scholaris* bark. Hence, the present study was directed towards evaluation of the antiplasmodial activity of *Alstonia scholaris* leaf extract, *in vitro*.

2. METHODS AND MATERIALS

2.1 Preparation of Crude Plant Extracts

The *Alstonia scholaris* leaves were collected from Navarangpura area, Ahmedabad and authenticated by Professor A. U. Mankad (GUJBOT/A/T/19/ *Alstonia scholaris*), Botany Department, Gujarat University, Ahmedabad, India. Leaves were cleaned with single distilled water and air-dried in the shade at the environmental temperatures (27°C–37°C) and the leaves were powdered mechanically using a commercial electrical stainless steel blender. The powder was defatted with an automated solvent

extractor using chloroform. The defatted powder was further extracted using methanol: water (70:30) and water in a soxhlet extractor. The extracts were concentrated, dried and stored at 4°C for further studies [13].

2.2 Phytochemical Studies

Qualitative analysis for determining the presence of alkaloids, tannins, flavonoids, terpenoids, glycosides and saponins in the plant extracts, was carried out using standard methods as described by Harborne [13], with some modifications [14]. The dried extracts were dissolved (3 mg/ml concentration) in respective extraction solvent, filtered and used for various qualitative tests.

2.3 High Performance Thin Layer Chromatography (HPTLC)

Both the aqueous and hydro-alcoholic extracts were developed with various solvent systems in TLC for the selection of the best solvent system for HPTLC studies. Samples (10 µL) were spotted as 6 mm bands, starting 15 mm from the edge of the plates, by means of a Camag Linomat V sample applicator and the plates were developed to a distance of 80 mm above the position of sample application in a Camag twin-trough chamber previously saturated with two different mobile phase for 30 minutes. The mobile phase were ethyl acetate: glacial acetic acid: formic acid: distilled water (100:11:11:26) and Ethyl acetate: methanol: water (81:11:08). Densitometric evaluation of the plates was performed at λ = 366 nm using a Camag Scanner III with tungsten lamp in conjunction with WINCATS III software for quantification [15,16].

2.4 Antioxidant Assay by DPPH

The antioxidant activity of the each extract was assessed by the ability of the extract to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals according to the method reported by Gyamfi et al. [17]. 50 µl of the plant extract in methanol, yielding 250 µg/ml with double dilution series respectively in each reaction were mixed with 1 ml of 0.1 mM DPPH in methanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50µl) only was used as control of experiment. After 30 minutes of incubation at room temperature the reduction of the DPPH free radical was measured reading the absorbance at 517 nm. Ascorbic acid was used as the standard compound.

The percent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = (\text{Abs of control} - \text{Abs of test sample} / \text{Abs of control}) \times 100$$

2.5 *In vitro* Study of Erythrocyte Membrane Stabilization

The membrane stabilizing activity of both extracts of leaves of *Alstonia scholaris* was assessed using the haemolysis assay by Jansen et al. [18]. Red blood cells suspensions (10% in PBS (v/v) were incubated under agitation at room temperature for one hour with extract or pure compound solutions from 1000µg/ml with serial dilution up to 7.18 µg/ml concentration. The mixtures were then centrifuged at room temperature for 5 minutes at 10,000 × g and the absorbance of the supernatants was measured at 550 nm with a microplate reader. The crude extracts were tested in triplicate at a final concentration of 1000 µg/ ml. Triton X-100 1% (v/v) was used as a positive control (100% red blood cell lysis) and PBS as a negative control (0% red blood cell lysis). The red blood cell lysis percentage (H) was determined as follows:

$$H = [(\Delta A550 \text{ nm sample} - \Delta A \text{ 550 nm PBS}) / (\Delta A \text{ 550 nm Triton X-100 1\%} - \Delta A \text{ 550 nm PBS})]$$

Aqueous and Hydro-alcoholic extract (1 mg/ml) of leaves *A. scholaris* were dissolved in 5% DMSO in RPMI 1640 media and used for this assay.

2.6 *In vitro* Cultivation of *Plasmodium falciparum*

Chloroquine (CQ)-sensitive strain MRC-2 and CQ-resistant strain RKL-9 of *Plasmodium falciparum*, obtained from NIMR, New Delhi, were used for *in vitro* blood stage culture to test the antiplasmodial efficacy of different plant extracts. *Plasmodium falciparum* culture was maintained according to the method described by Trager and Jensen [19] with minor modifications, in fresh O^{+ve} human erythrocytes suspended at 5% haematocrit in RPMI 1640 (Himedia) medium with 25 mM HEPES containing 0.2% sodium bicarbonate, 10% O^{+ve} treated plasma and 50 µg/L gentamicin and incubated at 37°C in CO_2 incubator. Every day, infected erythrocytes

were transferred into fresh complete medium and erythrocytes to propagate the culture.

2.7 *In vitro* % Inhibition of Entry of Parasites

In vitro percent inhibition of entry of parasites calculation was determined by Jonvile et al. [20] with some modifications [21]. Normal RBCs were treated with the plant extract (two-fold serial dilutions of each extract) for 24 hours and exposed to infected RBCs (iRBCs) with 2% parasitemia with early ring stages. Normal RBCs without treatment with plant extracts were considered as positive control. The plates were incubated at 37ºC for 20-24 h, after confirmation of presence of mature schizonts in control wells, without drug. The blood from each well was harvested and a thin film was prepared. The films were fixed with methanol and stained with JSB I and JSB II (Malarial parasite stain). The numbers of schizonts of the control and treated samples were counted to assess the effect of the hydro-alcoholic extract. Growth inhibition was expressed as percent of the number of schizonts for each concentration, compared with untreated controls. Mean IC_{50} values were calculated from dose-response curves (percentage of schizonts vs. logarithm of drug concentration). All these values are expressed as percent inhibition of entry of parasites.

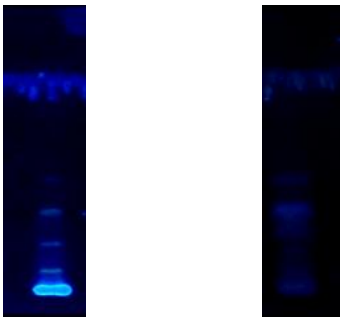
2.8 Lipid Peroxidation Assay

The method is based on the reaction of Thiobarbituric acid (TBA) with malonyl dialdehyde (MDA) and other breakdown products of peroxidised lipids collectively called as thiobarbituric acid reactive substances (TBARS). Thiobarbituric acid reactive species (TBARS) level in normal RBCs, infected RBCs (iRBCs) and RBCs treated with plant extracts determined by the method of Okhawa et al. [22].

3. RESULTS

3.1 Qualitative Phytochemical Analysis

Results obtained for qualitative phytochemical analyses for phytocomponents are given in Table 1. A higher amount of alkaloids and flavonoids are present in both the extracts followed by saponins, glycosides and terpenoids. No traces of tannins could be detected in both hydro-alcoholic and aqueous extracts. The study revealed that *Alstonia scholaris* showed best results in Ethyl Acetate: Glacial acetic acid: Formic acid: Water::100:11:11:26 (solvent system1) and Ethyl acetate: Methanol: Water:: 81:11:18 (solvent system2) solvent systems. The HPTLC images shown in Fig. 1 indicate that there are number of phytocompounds present in the extract. The R_f values ranged from -0.03 to 0.80 for solvent system1 and 0.02 to 0.97 for solvent system2. It is also clear from the chromatogram as shown in Fig. 2 A and B that 12 and 17 peaks for respective solvent systems. Thus, the data obtained reveals the abundance of phytoconstituents present in the hydro-alcoholic extract of *Alstonia scholaris* leaves.



Ethyl acetate: formic acid : acetic acid: water (100:11:11:28)

Ethyl acetate: methanol:water (81:11:08)

Fig. 1. Showing the chromatogram of hydro-alcoholic extract of *A. scholaris* leaves under 366 nm for corresponding solvent system

3.2 Antioxidant Assay

The data shown in Table 2 indicates the antioxidant activities of the hydroalcoholic and aqueous extracts of *Alstonia scholaris* using the DPPH radical scavenging assay with the concentration between 250-7.81 µg/ml. From the results of this assay, we could conclude that both the extracts had good antioxidant activity comparable to ascorbic acid at high concentration. Compared to the aqueous extract, hydro-alcoholic extract showed higher anti-oxidant activity (Table 2).

3.3 Erythrocyte Membrane Stabilization Assay

Table 3 shows the % haemolysis after treating the erythrocyte with different concentrations of the extract ranging from 7.81 to 1000 µg/ml in double dilution series. Before checking antiplasmodial activity of the extract, the effect of

extract on erythrocyte membrane was checked. The hydroalcoholic extract of *Alstonia scholaris* showed concentration dependent inhibition of erythrocytic haemolysis indicating that the plant contains constituents that protect the erythrocyte from haemolysis.

3.4 Lipid Peroxidation Assay

The results suggest a high level of lipid peroxidation in the cultured RBCs (Table 4). After treatment with the hydroalcoholic extract a significant decline was observed in the lipid peroxidation status as indicated from MDA formed.

3.5 Assay for Inhibition of *Plasmodium falciparum* Ingress

Table 5 shows the % inhibition of *Plasmodium falciparum* ingress into RBCs. The EC_{50} value for MRC2 is 8.87 µg/ml and for RKL9 is 34.27 µg/ml. The results were expressed as means ± SE with n = 3.

4. DISCUSSION

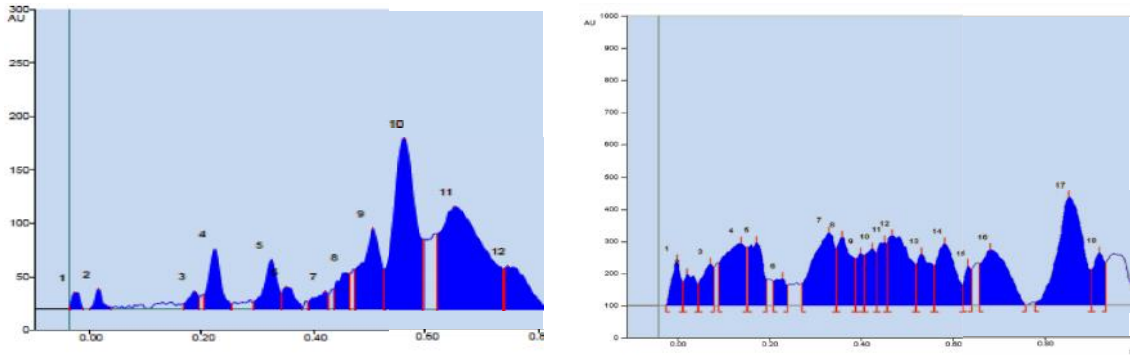
Malaria is a major public health problem in India though it is both a preventable and treatable disease. The development and spread of drug resistance strains of *Plasmodium falciparum* have limited the effectiveness of the currently used malarial drugs [23]. Therefore now these resilient *Plasmodium* parasites have triggered the search of new, efficient anti-plasmodial agents with minimal side effects. Natural products, including plants, animals and minerals have been the basis of treatment of human diseases [24] including malaria. Natural products and particularly medicinal plants have remained an important source of new drugs [5]. Keeping this in mind, the hydro-alcoholic extract of *Alstonia scholaris* was tested for erythrocyte membrane stabilization to prevent the entry of the parasite into the red blood cells so that spread of disease can be halted.

In the present study we have investigated phytochemical composition of leaves of *A. scholaris* along with their antiplasmodial and antioxidant properties. The analysis revealed higher quanta of alkaloids and flavonoids in the hydro-alcoholic and aqueous extracts. Glycosides, saponins and terpenoids are also present but tannins are absent in the extracts. These phytochemicals are known to possess a wide range of activities to help in protection against chronic diseases. The membrane stabilization potential demonstrated in this study by these phytocomponents may be due to the potent antioxidant activity against the actions of *Plasmodium* speices.

Table 1. Table showing the phytochemical constituents of the *Alstonia scholaris* leaf extracts

Extracts	Phyto- constituents					
	Alkaloids	Tannins	Flavonoids	Glycosides	Saponins	Terpenoids
Aqueous	+++	-	++	+	+	+
Hydro-alcoholic	++++	-	+++	++	++	+

(+ = present; - = absent)



(A) Ethyl acetate: formic acid : acetic acid: water (100:11:11:28)

(B) Ethyl acetate: methanol: water (81:11:18)

Fig. 2. Showing the area chromatograph of hydro-alcoholic of *A. scholaris* leaves under 366 nm for corresponding solvent system

Table 2. Showing DPPH scavenging activity of hydroalcoholic and aqueous extracts *Alstonia scholaris* leaf

	Concentration of the extract					
	250 μg/ml	**125 μg/ml**	**62.5 μg/ml**	**31.25 μg/ml**	**15.63 μg/ml**	**7.81 μg/ml**
Ascorbic acid	93.25±1.78	91.70±1.71	89.31±1.95	78.33±2.31	75.90±1.55	66.46±1.98
Hydro-alcoholic	83.40±1.43	78.78±0.26	62.35±0.59	54.81±0.54	43.96±0.43	21.19±1.00
Aqueous	79.75±0.34	62.65±0.45	59.56±0.32	50.61±0.60	29.05±0.85	17.98±0.53

Values are Mean ± S.E. n=6

Table 3. Showing % haemolysis activity of hydroalcoholic extract of *Alstonia scholaris* leaf

Concentration	**1000 μg/ml**	**500 μg/ml**	**250 μg/ml**	**125 μg/ml**	**62.5 μg/ml**	**31.25 μg/ml**	**15.63 μg/ml**	**7.81 μg/ml**
% Hemolysis	3.59±0.11	4.3±0.15	4.94±0.35	6.65±1.74	9.29±0.14	10.25±0.08	12.27±0.13	16.27±0.15

Values are Mean ± S.E. n=6

Table 4. Showing LPO level in control, infected and treated RBCs

RBC	LPO ($\times 10^4$n moles of MDA formed/100 mg of tissue wt/60 mins)	
	MRC-2	RKL-9
Control RBC	0.158±0.02	0.158±0.02
Infected RBC (iRBC)	0.949±0.01**	1.007±0.01**
iRBC+ Hydro-alcoholic extract	0.21±0.02*	0.352±0.03**

*Values are Mean ± S.E.; ** P<0.001*

Table 5. Showing % inhibition of *Plasmodium falciparum* ingress into RBCs

Concentration (µg/ml)	MRC 2	RKL 9
3.91	28.21±0.90	11.72±0.84
7.81	42.23±0.60	21.57±1.46
15.63	56.18±1.25	28.56±1.76
31.25	60.98±0.74	44.8±0.69
62.5	62.75±0.30	56.26±0.96
125	66.07±0.17	56.9±0.90
250	78.19±0.25	66.12±0.67

Values are Mean ± S.E

DPPH radical scavenging activity is one of the most widely used method for screening the antioxidant activity of plant extract. Preliminary phytochemical analysis in the present study has shown the presence of flavonoids, terpenoids in the aqueous and hydro-alcoholic extracts, which has been already recognized for its free radical scavenging activity and regulate various oxidative reactions occurring naturally, it can be correlated with the reported data [25,26].

Compared to normal human red blood cells (RBCs), infected RBCs (iRBCs) have dramatically altered structural and functional properties that include decreased deformability and the appearance of electron dense protrusions, or knobs, at the RBC membrane that mediate binding to the vascular endothelium [27] which facilitate entry of the parasite and its propagation inside the erythrocytes. Similar to *in vitro* studies on the *Lantana camara* extract [21], we can say that the hydro-alcoholic leaf extract showed no significant effect on integrity of red blood cells.

Recent studies carried out by Idonije et al. [28] revealed that lipid peroxidation level of the infected patients is significantly higher than the malaria negative subjects. Earlier studies from our laboratory have also reported that *in vitro* cultured infected RBCs have higher lipid peroxidation in comparison to normal control RBCs [14]. Flavonoids and Phenolic compounds are the largest groups of phytochemicals that account for most of the antioxidant activity of plants or plant products [29], which are effective in bringing about a decline in lipid peroxidation. Consequently, the data indicates that the extracts have the potential to effectively control the reactive oxygen species.

It was observed that the *Alstonia scholaris* hydro-alcoholic extract did not show hemolysis on human erythrocytes between 1000 to 7.18 µg/ml plant extract concentrations. The results of the present study thereby reveal that *Alstonia scholaris* hydro-alcoholic leaf extract holds immense promise as a potent antiplasmodial agent. This experiment demonstrates that exposure to extract modifies the erythrocyte membrane and reinforces it such that the RBC become unsuitable as parasite host cells or the entry of the parasite into the erythrocyte is halted even in the absence of extract in the growth medium.

5. CONCLUSION

From this study, it was observed that *Alstonia scholaris* possesses significant antiplasmodial activity against both MRC 2 and RKL 9 *Plasmodium falciparum* strains. The extract has the ability to protect human RBCs against lysis providing support to claims that either the extract made certain molecular modification to the RBCs, that could prevent entry of the parasite in to it or the red blood cell becomes an unsuitable host to the parasite for their propagation. Though, further *in vivo* evaluation is needed to support the potency of the extract of *Alstonia scholaris*, the hydro-alcoholic extract of *Alstonia scholaris* could be a useful alternative in controlling the parasite proliferation. Hence, this research would have significant clinical implications since this plant could be an effective anti-malarial agent which could be used alone or in combination, to effectively control this dreaded parasite protozoan.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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