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Lantana camara L. Aqueous-methanolic Extract Provides Potent Red Blood Cell Membrane Fortification against Plasmodial Attack

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LBG and HNH designed the study, performed the statistical analysis, wrote the protocol and authors UUJ and SDG wrote the first draft of the manuscript. Authors UUJ, DVJ and SDG managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of our study was evaluation of the possible anti-plasmodial efficacy of *Lantana camara* L. extracts, 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- 2015.
Methodology: In the present study, we have tested the *In vitro* anti-plasmodial activity of aqueous-methanolic extract (fraction VIII) of leaves of *Lantana camara* L. against MRC-2 (CQ-sensitive) and RKL-9 (CQ-resistant) strains of *Plasmodium falciparum*. The cytotoxicity test on HeLa cell line was evaluated using the 3-[4, 5-dimethylthyazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) test in order to determine the selectivity index.
Results: According to the results, the aqueous-methanolic and aqueous extracts of leaves of *Lantana camara* L. manifested potent anti-oxidant activity. The fraction VIII of aqueous-methanolic extract showed 48.0±0.12% and 35.0±021% (MRC-2 and RKL-9 respectively) inhibition of entry of parasite in the RBCs at 7.81 µg concentration. Qualitative tests revealed the presence of various

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phytocomponents in the leaves of *Lantana camara* L. that may be responsible for the *In-vitro* activity of the plant.
Conclusion: On the basis of the study it can be concluded that leaves of *L. camara* L. are rich sources of a vast quanta of secondary metabolites/ phytocomponents which can provide leads to a potent prophylactic drug against malaria.

Keywords: Antiplasmodial; Lantana camara L.; antioxidant; erythrocyte membrane stabilization.

1. INTRODUCTION

Malaria remains one of the major causes of human mortality worldwide. It is rampant in the tropical areas of Asia, Africa, and Central and South America. According to the latest estimates, 198 million cases of malaria occurred globally in 2013 (uncertainty range 124–283 million) and the disease led to 5, 84,000 deaths (uncertainty range 367 000–755 000). According to the World Malaria Report 2014, 22% (275.5 m) of India's population live in high transmission (> 1 case per 1000 population) areas, 67% (838.9 m) live in low transmission (0–1 cases per 1000 population) areas and 11% (137.7 m) live in malaria-free (0 cases) areas. In 2013, 0.88 million cases have been recorded, with 128 million tests being conducted on the suspected cases, with *P. falciparum* causing 53% and *P. vivax* causing 47% of the infections. The incidence of malaria in India accounted for 58% of cases in the South East Asia Region. At present, official figures for malaria in India indicate 0.7–1.6 million confirmed cases and 400-1,000 deaths annually [1].

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoans belonging to the genus *Plasmodium.* The disease is transmitted most commonly by an infected female *Anopheles* mosquito. The main *Plasmodium* species, which mainly infect mammals especially humans are: *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi.* Infection with *P. falciparum*, if not promptly treated, can quickly progress to cerebral malaria, caused specifically by *P. falciparum* which can prove fatal.

It has been found that natural products continue to play an important role in the discovery and development of new pharmaceuticals, as clinically useful drugs. Herbal extracts from medicinal plants have immense therapeutic potential, are cost effective and have less side effects as well as they are the richest source of primary and secondary metabolites having potent biological activities.

Lantana camara L belongs to the family Verbenaceae [2]. Different parts of *L. camara* L. are reported to possess essential oils, phenolic compounds, flavonoids, carbohydrates, proteins, alkaloids, glycosides, iridoid glycosides, phenyl ethanoid, oligosaccharides, quinine, saponins, steroids, triterpenes, sesquiterpenoides and tannin as major phytochemical groups. Extracts from the *Lantana* leaves exhibit antimicrobial [3], insecticidal [4] and nematicidal activity and also contain verbascoside, which possesses antimicrobial, immunosuppressive and antitumor activities. Lantanoside, linaroside and camarinic acid have been isolated and are being investigated as potential nematocides. *Lantana* oil is sometimes used for the treatment of skin itches, antiseptic for wounds, leprosy and scabies [5].

Keeping these points in mind and based on the abundant phytochemical resources in *Lantana camara* L. the present study was directed towards evaluating its possible anti-plasmodial efficacy, with specific emphasis on its role in stabilization of the RBC membrane.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Plant Materials

The leaves of plant *Lantana camara* L. was collected from the School of Sciences campus, at Gujarat University, Ahmedabad in the month of December 2014, and was authenticated by the Botany Department, School of Sciences, at Gujarat University with voucher specimen submitted to the herbarium.

The leaves were cleaned thoroughly with single distilled water and shade dried, after which they were powdered and stored in an airtight container. The powder was sieved and defatted with Petroleum ether (60℃) for 24 hours at room temperature by continuous shaking. 50 gm defatted powdered, material was extracted at 50℃ with a volume of 500 ml comprising of two

different solvents 1) aqueous and 2) aqueous-methanolic (70:30 methanol:water) in the soxhlet apparatus for three days till the colour in the siphon became colourless. The extracts were dried at 60℃ in the oven and the yields collected after drying were weighed and kept at 4℃ for further analysis [6,7].

2.1.1 Preliminary phytochemical screening tests

The aqueous and aqueous-methanolic extract of the powdered plant material were subjected to various qualitative tests for the detection of presence of various phytochemical constituents [8].

2.1.2 High Performance Thin Layer Chromatography (HPTLC)

The aqueous and aqueous-methanolic extracts were further subjected to HPTLC for the conformation of the active constituents. 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 mobile phase for 30 minutes. The mobile phase was ethyl acetate: glacial acetic acid: formic acid: distilled water (100:11:11:26). 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 [9].

2.1.3 Preparative TLC of extract

Glass plates measuring 20 cm x 10 cm were used for the preparative thin layer chromatography (TLC) and silica powder $60F_{254}$ was used as the adsorbent. The crude extract was dissolved in the solvent of extraction i.e. methanol: water; (70:30) and spotted with the aid of capillary pipettes. The spots were allowed to dry in air for five minutes before developing in the solvent tanks which were saturated with the solvent system for 30 minutes. The tanks were sealed and allowed to stand undisturbed until the solvent front traversed the length of the plate to 1 cm of the top of the coated portion of the plates. The plates were removed and the solvent front marked with a pencil. Spots on the developed plates were detected using a UV source of wavelength 366 nm. The bands were scraped out and dissolved in methanol and the mixture was allowed to stand for thirty minutes. The mixture was filtered and fractions were labelled, as fractions I-VIII. The fractions were stored at 4℃ in refrigerator until further analysis.

2.1.3.1 In-vitro Experimental test series

The following plant extracts were evaluated *in-vitro:*

1. LAQ : *L. camara* leaves aqueous extract.
2. LHA : *L. camara* leaves aqueous-methanolic extract (Fraction VIII).

2.2.3.2 The in-vitro experimental tests carried out included:

RBCs : Normal RBCs in culture.
iRBCs : RBC infected with *Plasmodium falciparum.*
RBCs + LAQ: RBCs were treated with 7.81-250 µg/ml *L. camara* L aqueous extract.
RBCs + LHA (Fraction VIII): RBCs were treated with 7.81-250 µg/ml *L. camara* L aqueous-methanolic extract (Fraction VIII).

The Red Blood Cells (RBCs) were obtained from volunteers of a recognised Blood Bank, after the ethical clearance from the Institute Human Ethical Committee, Department of Zoology, Gujarat University.

2.2 Antioxidant Assay by DPPH

The antioxidant activity of the extracts was evaluated by DPPH Antioxidant assay, which was originally described by Blois [10].

2.3 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 were determined by the method of Okhawa et al*.* [11].

2.4 *In Vitro* Study of Erythrocyte Membrane Stabilization

The membrane stabilizing activity of both extracts of leaves of *L. camara* L was assessed using the haemolysis assay by Jansen et al*.* [12].
Preparation of plant extracts:

Aqueous extract (1 mg/ml) of leaves *L. camara* L was dissolved in 5% DMSO in RPMI 1640 media and used for this assay.

Fraction VIII of the aqueous-methanolic extract (1mg/ml) of leaves *L. camara* L was dissolved in 5% DMSO in RPMI 1640 media and used for this assay.

2.5 *In Vitro* % Inhibition of Entry of Parasites

In vitro percent inhibition of entry of parasites was determined by Jonvile et al. [13] with some modifications. Normal RBCs were treated with the plant extracts for 24 hours and exposed to iRBCs with 2% parasitemea. Normal RBCs without treatment with plant extracts were considered as control. The numbers of schizonts of the control and treated samples were counted to assess the consequence of the aqueous and Fraction VIII of aqueous-methanolic extract. All these values are expressed as percent inhibition of entry of parasites.

2.6 3- (4, 5-Dimethyl thiazol-2-YL)-2, 5-Diphenyl tetrazolium bromide (MTT) Assay

This colorimetric assay has been done in order to measure the cytotoxicity of the crude extracts of the plant chosen. It was determined on the HeLa cell lines by the method described by Mosmann [14] and Wilson [15].

3. RESULTS AND DISCUSSION

Since for a very long time *L. camara* L has been used in traditional medicinal systems for the treatment of itches, cuts, ulcers, swellings, bilious fever, cataract, eczema and rheumatism. Various parts of the plants are used in the treatment of colds, headache, uterine haemorrhage, chicken pox, eye injuries, whooping cough, asthma, bronchitis and arterial hypertension.

In the present investigation therefore *L. camara* L leaf extracts were tested for their possible *in vitro* action on RBC membrane which could lead to exclusion of *Plasmodium* attack.

Preliminary phytochemical analysis has revealed that these leaf extracts were a rich source of potent phytochemicals, results are given in Table 1. In the aqueous extract, the presence of phenolics, flavonoids, carbohydrates, alkaloids, glycosides and tannins have been observed. The aqueous-methanolic extract was shown to have the following phytoconstituents shown in the order of all the tested tested groups of phytochemicals ≥ Phenolics ≥ Alkaloids ≥ Flavonoids ≥ Steroids ≥ Triterpenoids > Glycosides ≥ Carbohydrates > Tannins > Saponins (Table 1).

Table 1. Qualitative analysis showing the presence or absence of various phytochemicals in the leaves of *Lantana camara*

No.	Compounds	Aqueous extract	aqueous-methanolic extract
1	Alkaloids	+	+++
2	Glycosides	+	++
3	Tannins	+	+
4	Saponins	-	+
5	Flavonoids	++	+++
6	Carbohydrates	++	++
7	Steroids	-	+++
8	Triterpenoids	-	+++
9	Phenolic compounds	+++	+++

(+) present (-) absent

The presence of alkaloids may enhance plant metabolic processes. Triterpenes are known to express cytotoxic activity against a wide range of organisms, ranging from bacteria to fungi. Saponins, which are glycosides of triterpenes or steroids and include the group of cardiac glycosides and steroidal alkaloids, are often prime components in traditional medicines as anti-infective agents.

Both the extracts of *Lantana camara* L. leaf showed almost similar peaks and Rf values in the HPTLC analysis. Fig. 1 demonstrates the varied phytochemical constituents in the aqueous and aqueous-methanolic extracts as obtained under 254 nm and 366 nm respectively. The corresponding HPTLC chromatograms are presented in Figs. 2 and 3.

Though both the extracts had shown potent effects against *Plasmodium*, the effect of aqueous-methanolic extract was more pronounced. The (fraction VIII) peak observed for the aqueous-methanolic extract showed greater area than the other peaks of the aqueous extract. Therefore only fraction VIII of aqueous-methanolic extract was selected for further studies and this fraction was collected by preparative TLC.

Specific secondary metabolites of plants have developed capability to interact with molecular targets affecting the cells, tissues, and physiological functions in competing microorganisms, plants and animals as a mechanism to promote the ecological survival of plants, and therefore, these phytochemicals are key candidates contributing to the medicinal value of the plant [16].

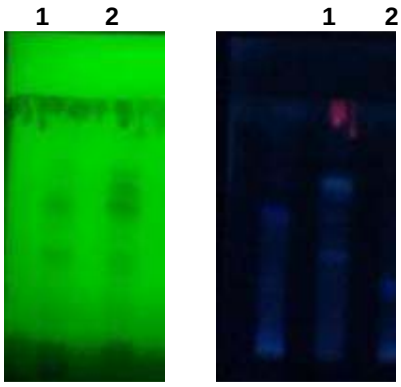

Fig. 1. Showing the chromatogram under UV 254 nm and 366 nm

Lane 1 Aqueous extract and Lane 2 Aqueous-methanolic extract

3.1 Antioxidant Activity

Antioxidant activity of both the extracts was determined by DPPH radical scavenging assay and the result obtained showed the antioxidant potential of plant with varying degrees according to the concentration mentioned in Table 2.

Phytochemicals, especially plant phenolics constitute a major group of compounds that act as primary antioxidants. They can react with active oxygen free radicals, such as hydroxyl radicals superoxide anion radicals and lipid peroxy radicals, and inhibit lipid oxidation at an early stage [17].

It was also observed in this study that both the extracts manifested potent anti-oxidant activity. In correlation, phytochemical analysis revealed presence of several effective anti-oxidant components. Flavonoids and tannins are phenolic compounds, which are a major group of phenolic compounds that act as primary antioxidants or free radical scavengers [8] and hence mainly phenolics, flavanoids and tannins are thought to be responsible for free radical scavenging effect. It is claimed that phenolic compounds are powerful chain breaking antioxidants [18]. Hateno et al. [19] have attributed the scavenging activity of phenolic group is due to its hydroxyl group. Both aqueous (75.13%) and aqueous-methanolic (85.23%) extracts of leaves showed a strong inhibition of DPPH radical at the concentration of 250 µg/ml, substantiating its antioxidant potential.

3.2 Lipid Peroxidation Assay

The study revealed that the infected RBCs in culture manifested a high level of lipid peroxidation. The results however indicated that there was a significant decrease in lipid peroxidation in the cultured infected RBC, after treatment with the extracts (Table 3). This decrease could be attributed to the potent antioxidant activity of these extracts.

Lipid peroxidation was found to be significantly high in the infected RBCs ($p<0.001$), when compared to the normal RBCs. Lipid peroxidation, which is widely recognized as primary toxicological event, is caused by the generation of free radicals from a variety of sources including organic hydro-peroxides, redox cycling compounds and iron-containing compounds. Becker et al. [20] have shown an increase in the lipid peroxidation of *Plasmodium* infected RBCs. Moreover, Erel et al*.* [21] have demonstrated that *plasmodia* succeed in accumulating free radical scavenging enzymes within their own cells but deplete them in red blood cells of the host. After treatment with the both the extracts the lipid peroxidation significantly reduced to the normal range. Polyphenols (flavonoids) have been known to effectively restrict free radical induced peroxidation of lipid. According to Verstracten et al. [22] in addition to their protein binding and direct scavenging activity, these potent antioxidants interact with membrane lipids and prevent the access of deleterious molecules across the cell membrane. In this study, both extracts were capable of preventing the formation of MDA in a dose dependent manner. This assay revealed that the extracts might prevent reactive free radical species from damaging biomolecules such as lipoprotein, DNA, amino acids, sugar, proteins and PUFA in RBC membrane.

3.3 Erythrocyte Membrane Stabilization Assay

Membrane stabilizing profiles of various extracts of *L. camara* L on bovine red blood cells exposed

to both heat and hypotonic induced lysis were reported previously [23]. The results of percent erythrocyte membrane stabilization assay have been given in Table 4.

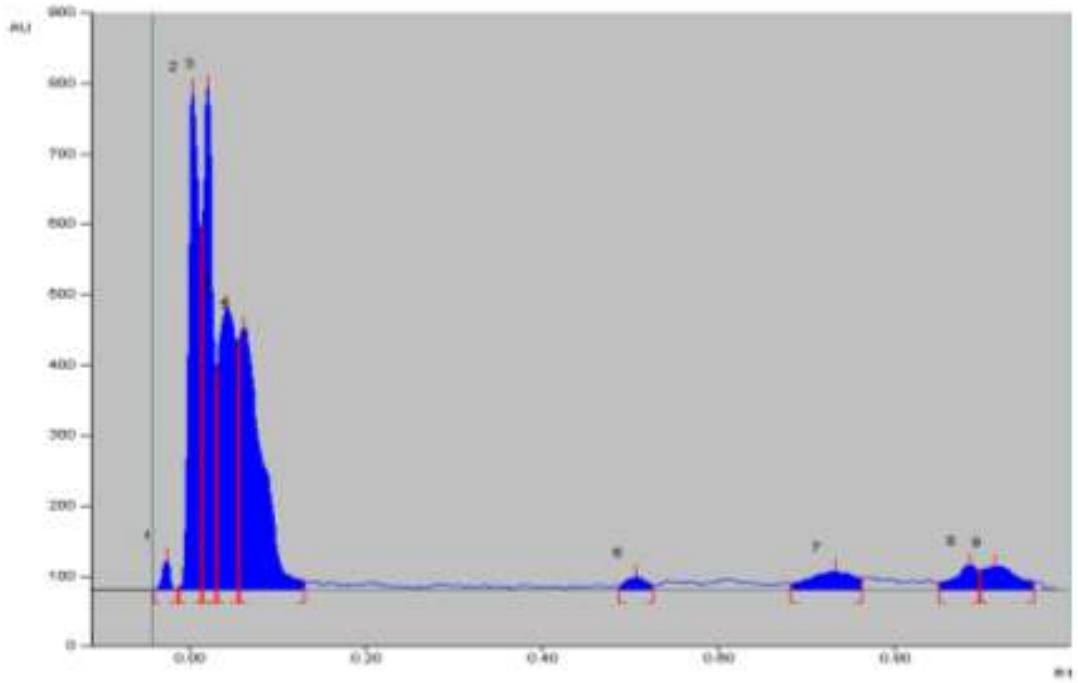

Fig. 2. Showing the HPTLC chromatogram area and peaks of the aqueous extract of *Lantana camara* L using Win CATS evaluation software (Version 1.4.6.8121) at 366 nm

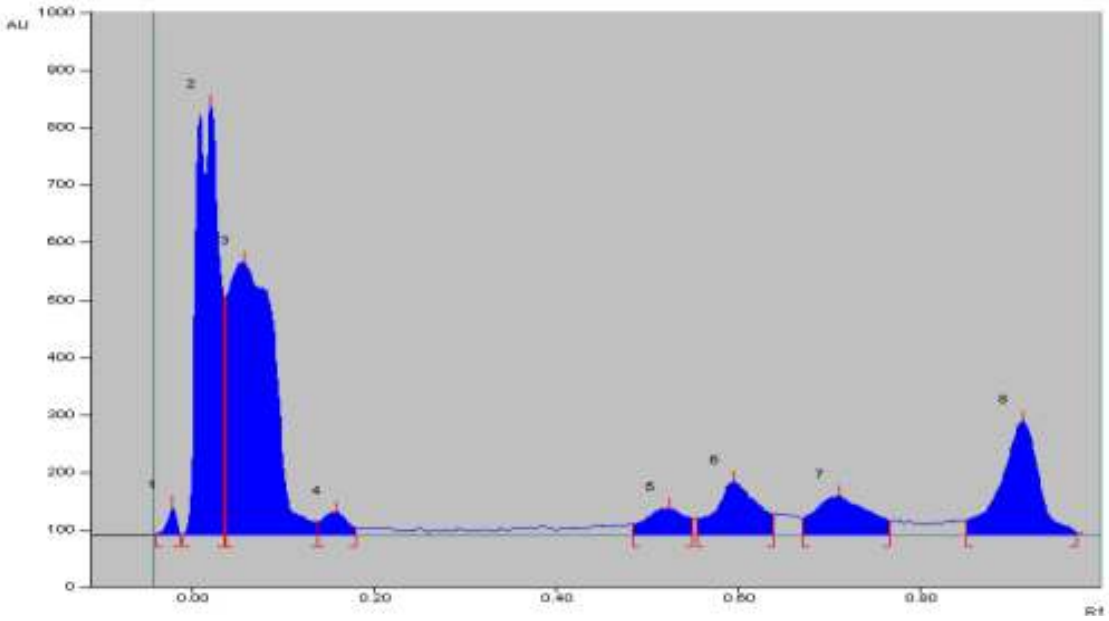

Fig. 3. Showing the HPTLC chromatogram area and peaks of the aqueous-methanolic extract of *Lantana camara* L using Win CATS evaluation software (Version 1.4.6.8121)

Table 2. Showing the percent anti oxidant activity of the both extracts of leaves of *L. camara* L

	Concentration of the extracts					
	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	82.12±2.31	78.33±1.55	75.90±1.98
Aqueous-methanolic (fraction VIII) extract	85.23±1.36	81.75±0.42	79.04±0.68	77.36±0.66	75.22±0.57	73.24±0.69
Aqueous extract	75.13±1.43	73.54±0.26	70.40±0.59	64.51±0.54	62.80±0.43	44.42±1.00

Table 3. Showing lipid peroxidation activity in control RBCs, iRBCs, and treated with both the extracts of *L. camara* L

RBC	**LPO(×10^{4}n moles of MDA formed/100mg of tissue wt/60 mins) (MRC-2)**	**LPO(×10^{4}n moles of MDA formed/100mg of tissue wt/60 mins) (RKL-9)**
Control RBCs	0.158±0.02	0.158±0.02
iRBCs	0.949±0.01**	1.45±0.03**
iRBCs+HA(fraction VIII)	0.51±0.01**	0.67±0.02**
iRBCs+ AQ	0.69±0.023	0.78±0.02

Values are Mean ± S.E. *** p<0.001*

We observed a decline in the entry of the parasites in relation to the concentration of the fraction VIII of aqueous-methanolic extract. Earlier studies have shown that very few herbal drugs are capable of stabilizing the red blood cell membrane [24,25]. The mode of action of the extracts could be connected with binding to the erythrocyte membranes with subsequent alteration of the surface charges of the cells. This might have prevented physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the hemolysis of red blood cells. It has been reported that certain saponins and flavanoids exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins and saponins possess ability to bind cations, there by statbilizing erythrocyte membrane and other biological macromolecules [24]. Membrane stabilizing profiles of various extracts of *Lantana camara* on bovine red blood cell exposed to both heat and hypotonic solutions, induced lyses were reported previously [23]. To check the use of these plant extracts as potent antimalarial drugs we carried out the membrane stabilizing activity assay on human erythrocyte. The leaf extracts showed high potent membrane stabilizing activity, which subsequently inhibited entry of the *Plasmodium*.

3.4 Anti-plasmodial Activity

The various parasite proteins (RESA, PfEMP1, PfEMP3 & KAHRP) expressed at different stages of parasite development that interact with red cell membrane have been identified [26]. Merozoites of *P. falciparum* including EBA175, EBL-140 for which cognate receptors on red cells (glycophorin A, B & C) respectively have been identified associated with the invasion process [27]. Apart from the involvement of individual red cell surface molecules in the invasion process, it is now becoming clear that the overall arrangement of surface molecules is also important. According to the data obtained in the present study the fraction VIII proved to have effective antiplasmodial activity. Since this fraction is native to the aqueous-methanolic extract, certain unknown component may exist in the extract which may be involved in the binding and modifications of the receptors of erythrocytes and therefore have an important role in the invasion of *Plasmodium* into RBCs. This may be main mechanism for the prevention of entry of parasites into the treated RBCs, and hence *Lantana camara* L. extracts show promise as a potential anti-plasmodial agent.

3.5 MTT Assay

It was found that there were no cytotoxic effects with increasing concentration on HeLa cell line from 7.81 µg to 250 µg concentration. Compared to the untreated HeLa cells, 96 to 98% growth and cell proliferation is maintained even in the treated cells. The results are tabulated in Table 6.

Table 4. Showing percent of erythrocytes membrane stabilization (Erythrocytes taken from normal blood sample)

	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**
Aqueous extract	91.0±0.49	90.4±0.32	89.17±.17	88.27±0.20	86.2±0.75	83.65±0.18
Aqueous-methanolic (fraction VIII) extract	97.52±0.17	96.88±0.24	94.83±0.26	92.46±0.17	91.47±0 .20	85.6±0.31

Values are Mean ± S.E

Table 5. % Inhibition of entry of parasites into the RBCs

Drug conc. (µg/ml)	iRBCs (No. of parasites)		% inhibition	
	MRC-2	**RKL-9**	**MRC-2**	**RKL-9**
Control	200	200		
3.91	114	130	43.0±0.12	35.0±0.21
7.81	104	120	48.0±.0.05	40.0±0.08
15.63	97	100	51.5±0.07	50.0±0.19
31.2	74	95	63.0±0.28	52.5±0.05
62.5	49	70	75.5±0.16	65.0±0.17
125	28	60	86.0±0.04	70.0±0.06
250	27	54	86.5±0.21	73.0±0.11

Values are Mean ± S.E

Table 6. Showing the percent growth proliferation of the both extracts of *L. camara* L on the HeLa cells

	Concentration of the extracts					
	250 µg/ml	**125 µg/ml**	**62.5 µg/ml**	**31.25 µg/ml**	**15.63 µg/ml**	**7.81 µg/ml**
Aqueous-methanolic (fraction VIII)	98.46±0.90	97.79±1.09	97.88±0.43	97.56±0.90	96.90±1.24	98.12±0.51
Aqueous	96.86±1.2	97.24±0.67	97.17±0.41	97.08±1.15	97.53±0.77	98.08±0.54

Values are Mean ± S.E

4. CONCLUSION

Herbal plants and its products have been valued for their medicinal significance and are known to have potent phytochemical and pharmacological action. The above mentioned research provides evidence of the medicinal properties of *Lantana camara* L., in relation to its role in stabilizing the RBC membrane and blocking entry of *Plasmodium* parasites into the cell.

From the present study, it could be concluded that the different therapeutic properties of *L. camara* are due to the various phytochemicals and secondary metabolites present in the plant. We observed a variety of flavanoids in the plant under study by the HPTLC analysis. We have specifically selected only fraction VIII of the aqueous-methanolic extract as other components are already present in the aqueous extract.

Both extracts of *L. camara* leaves had shown positive results for the effect on erythrocyte membrane stabilization. They could inhibit the entry of parasites, also showed effective antioxidant property and had no toxic effect due to certain unknown compounds in the crude extracts on the normal cells, which were proved by the MTT assay.

In comparison to the synthetic products that are regarded as unsafe to human life and environment. The phytochemicals identified in *Lantana camara* L. are relatively safe as demonstrated by the Phytochemical and MTT assay studies have been conducted on *L. camara.* L. On the basis of the results obtained

in the study, it can be inferred that the leaves of *L. camara* L. are rich sources of secondary metabolites/phytochemicals which can be used as a prophylactic agent against malaria.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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