

Antiplasmodial Potential of Traditional Phytotherapy of Some Remedies Used in The Treatment of Malaria in India

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Research

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Abstract

Background: The increasing problem of drug resistance to most of the available antimalarial therapies is a recurring issue. To overcome this issue, new antiplasmodial drugs are needed. Many plants have been used for treatment of malaria by traditional healers in India. The purpose of this research was to evaluate seven medicinal plants used against malaria by indigenous people in India, validate their use and identify the plant with the best antiplasmodial properties.

Methods: Methanol, hydro alcoholic (60:40) and aqueous extracts from seven ethnomedicinal plants were screened on chloroquine-sensitive (MRC-2) and chloroquine-resistant (RKL-9) strain of *Plasmodium falciparum* by schizont maturation inhibition assay and inhibition of *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH) activity.

Results: Out of 21 extracts, hydro alcohol extract of *B. variegata* showed the highest inhibition of schizont maturation with $81.77 \pm 0.96\%$ against MRC-2 and $78.08 \pm 3.76\%$ against RKL-9 strain. In *Pf*LDH inhibition assay, Methanol and hydro alcohol extract of *B. variegata* showed moderate antiplasmodial activity with IC_{50} 24.65 μ g/mL and 39.42 μ g/mL, respectively against MRC-2 strain. Methanol extract of *B. variegata* also exhibited moderate activity with IC_{50} 28.71 μ g/mL against RKL-9 strain.

Conclusion: The present study provided scientific evidence for indigenous Indian medicinal plants as a source of antiplasmodial agents. This finding supports the continued exploration of traditional medicines in the search for new antimalarial agents. The compounds responsible for the observed antiplasmodial effects in *B. variegata* are under investigation.

Background

Malaria still poses a threat to the health of residents and travellers in tropical and subtropical countries. There were 405 000 malaria deaths recorded globally in 2018 as per World Health Organization. Children aged below 5 years were more affected, as they accounted for 67 percent of malaria death globally in 2018. The African Region accounted for the highest, 94 percent of malaria deaths in 2018 [1]. Two of the main reasons for malaria perseverance are increasing *P. falciparum* drug resistance and insecticide resistance of mosquito vectors [2]. Amongst the five existing species of Plasmodium causing malaria in humans, *P. falciparum* is the most virulent and widespread infectious due to the resistance of the parasite to most of the available antimalarial drugs [3]. The development of resistance to golden therapeutic drugs like chloroquine and controlled use of new artemisinin analogs have created an urgent need to discover new antimalarial agents [4]. Artemisinin resistance has been reported first from clinical cases on the Cambodia–Thailand border and then spread to other countries [5]. The need for new drugs, preferably with new modes of action is therefore needed [6]. Plant species are the best source for chemotherapeutic prophylactics for malaria treatment [7]. Two most available medicine quinine and artemisinin invented from the scientific study of traditional remedies of various cultures. Many plant species are reported for

the treatment of malaria and fever [8]. Plants have been proven for their value as a rich source of a therapeutic molecule and many currently available drugs are natural products-derived compounds [9].

In this present study, the selection of plants to be screened for antimalarial activity was done based on the traditional claim of particular plants for the treatment of malaria. Here, we studied plants mentioned either in the traditional text or used by traditional healers of India for “*vishamjwara* (malaria)” and evaluated them *in vitro* for antimalarial activity.

Methods

Plant materials and chemicals

The plants were collected in the month of October-November from Saurashtra University Campus and the surrounding area, Rajkot. The parts of the plant used, their family, local name and traditional uses of the seven selected plants are stated in Table 1. The plant species were identified by Dr. Vrunda Thakar, Department of Bioscience, Saurashtra University, Rajkot. Voucher specimens were deposited to the Department of Pharmaceutical Sciences, Saurashtra University, Rajkot. The plant materials were dried under shade, stored in an airtight container at room temperature and powdered to 60 meshes when required.

RPMI-1640 media with phenol red, glutamine and HEPES buffer, albumax II, hypoxanthine, Sodium bicarbonate (NAHCO₃), sorbitol, Dimethyl sulphoxide (DMSO) and Giemsa stain were purchased from Himedia Laboratories. Quinine diphosphate and artemisinin were purchased from Sigma-Aldrich. All other chemicals were of analytical grade.

Preparation of crude plant extracts

25 gm dry powder of each plant species were extracted with methanol, methanol: water (60:40) and water by hot maceration at 60°C in a water bath for 6 h followed by 48 h maceration with continuous shaking in a rotary shaker at 100 RPM. This process was repeated three times with the same solvent. The extracts were filtered, combined and the solvent is removed using a rotary evaporator under vacuum. The dried extracts were stored at 4°C.

In vitro cultivation of *Plasmodium falciparum*

Chloroquine sensitive (MRC-2) and chloroquine-resistant (RKL-9) strain of *P. falciparum* were procured from the National Institute of Malaria Research (NIMR), New Delhi, India. Cultures were maintained *in vitro* by a modified method of Trager and Jenson [20] in human red blood cells 5% hematocrit using RPMI-1640 with phenol red, glutamine and 20 mM HEPES buffer as a culture medium supplemented with 0.4% albumax II, 0.0025% hypoxanthine, 0.21% NAHCO₃ and 40 µg/mL gentamycin. Slides of culture were observed after 6, 12, 24 and 48 h for regular development of parasite stages. Parasites were synchronized by repeated treatment of 5% sorbitol.

Schizont maturation inhibition assay

The antiplasmodial activity of extracts of the plants was assessed by the schizont maturation inhibition assay [21]. 100 µL of highly synchronized parasite culture with 1-1.5% parasitemia and 5% hematocrit was added in each well in 96 well plates along with 10µl of different extract of 10 and 30 µg/mL. Quinine diphosphate and artemisinin were used as positive control. Infected and uninfected erythrocytes were used as control and negative controls. Each concentration was repeated three times (triplicate each). The plates were kept for 48 h under 37°C, 5% CO₂ atmosphere. After 48 h of incubation, thin blood smears were prepared from each experimental and control wells on pre-labeled slides and fixed in methanol. Dried slides were stained with Giemsa stain and observe under 100 x under a microscope (LEICA DM1000 LED). Numbers of schizonts were counted per 200 asexual stages of parasites. The inhibition of schizont development in comparison to the control wells was determined.

Lactate dehydrogenase inhibition assay for *P. falciparum*

The antiplasmodial activity was analyzed by measuring Plasmodium lactate dehydrogenase (*Pf*LDH) activity [22, 23]. 100 µL of highly synchronous ring stage culture with 1% parasitemia and 3% hematocrit was incubated in 96-well microtitre plate with 10µL of different concentrations of test compounds (5-500 µg/mL) at 37 °C for 48 h. Quinine diphosphate and artemisinin were used as positive control. Infected and uninfected erythrocytes were used as control and negative controls. After incubation, plates were subjected to three 30 min freeze-thaw cycles, then 20 µL of supernatant were transferred to another microtitre plate containing 100 µL of Malstat reagent (0.125% Triton X-100, 130 mM L-lactic acid, 30 mM Tris buffer and 0.62 µM 3-acetylpyridine adenine dinucleotide; pH 9) and 25 µL of NBT-PES (1.9 µM nitro blue tetrazolium and 0.24 µM phenazine ethosulphate) solution per well. The plate was incubated in the dark for 30 min and absorbance was recorded at 630 nm using a microplate reader (BioTek ELx800). Control parasite cultures devoid of plant extracts or drugs were referred to as 100% *Pf*LDH activity. IC₅₀ values were calculated by the dose-response curve. Each analysis was performed in triplicate (n =3).

Statistical analysis

Results were expressed as means followed by a standard deviation. IC₅₀ was determined from the resulting dose-response curve non-linear regression using Prism 5 for Windows, Version 5.02 (Graph Pad Software, Inc) program. . Comparisons were made between disease control and treatments by using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests., with the level of significance set at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Results

Table 2 shows the herbarium number and the amounts of raw material used for extraction against the percentage yields of the extracts.

Schizont maturation inhibition

Twenty-one extracts were prepared from the seven plant species were first tested for schizont maturation inhibition assay at two concentrations 10 µg/mL and 30 µg/mL against Chloroquine sensitive (MRC-2) and chloroquine-resistant (RKL-9) strain of *P. falciparum* (Fig 1.). Parasites of all stages were observed in the control culture wells. Hydro-alcohol extract (30 µg/mL) of *B. variegata* showed more number of ring stages and with higher inhibition of schizont maturation with $81.77 \pm 0.96\%$ and $78.08 \pm 3.76\%$ against chloroquine-sensitive MRC-2 and Chloroquine-resistant (RKL-9) of *P. falciparum* respectively. The methanol extract (30 µg/mL) of *B. variegata* showed inhibition of schizont maturation with $76.40 \pm 1.69\%$ and $69.66 \pm 2.81\%$ against chloroquine-sensitive MRC-2 and Chloroquine-resistant (RKL-9) of *P. falciparum* respectively. Hydroalcoholic extract of *A. excelsa* and *F. arabica* also demonstrated interesting schizont maturation inhibition activity against both the strain. Microscopic examination of uninfected erythrocytes incubated with the extracts of these plants showed no morphological differences after 48 h of incubation.

Inhibition of PfLDH

All the extracts showed dose-dependent inhibition of *P. falciparum* by inhibiting PfLDH against both the strains. As per WHO guideline and some earlier research, [24] activity criteria for assay of plant extracts were defined as high when $IC_{50} < 10 \mu\text{g/ml}$, an IC_{50} 10-50 µg/mL; moderate activity, low activity when an IC_{50} between 50 - 100 µg/mL, and an $IC_{50} > 100 \mu\text{g/mL}$; lack of activity. Methanol and hydro alcohol extract of *B. variegata* showed moderate antiplasmodial activity with IC_{50} 24.65 µg/mL and 39.42 µg/mL respectively against MRC-2 strain. The methanol extract of *B. variegata* also exhibited moderate activity with IC_{50} 28.71 µg/mL against RKL-9 strain. The extracts were more active on MRC-2 than RKL-9 strain. IC_{50} of reference drug Quinine diphosphate and artemisinin for both the strain were stated in Table 3.

Discussion

Malaria is a highly destructive parasitic disease affecting many developing countries. Drug resistance to available therapy is a big challenge amongst all scientists. To overcome this resistance problem, traditional health care systems can be a better source for developing new molecules with more efficacies and minimum toxicity. Seven plant species were selected from the traditional system of India which has traditional use against malarial fever. Seven plant species have been tested first time for both *P. falciparum* strains. The present study reveals that leaves of *B. variegata* having the best activity amongst seven screened plants in schizont maturation inhibition assay and PfLDH inhibition assay. In the earlier report, some new secondary metabolites dihydrodibenzoxepins and flavanones were isolated from *Bauhinia* species and exhibited antimalarial activity (IC_{50} 5.8–11.2 µM) [25]. Phenolic metabolites and flavonoids seem to be the main active principles detected by *Bauhinia* spp. Leaves extract of *B. variegata* exhibited better schizonticidal action against *P. berghei* in mice screened by Peter's 4-day test [26]. The plant crude extracts screened were less active than reference drugs quinine and artemisinin, may be due to the composition of many heterogeneous compounds present with active compounds and active principles might show higher activity in their pure form. The screening results do not reflect an evaluation

of the traditional use of the plants. A majority of the plants selected did not show *in vitro* antiplasmodial activity, even though a strong correlation with malaria and its treatment in traditional medicine in India. This may be due to these plants may act as antipyretics or cure the symptoms of malaria, rather than having activity on malaria parasite. All the water extract tested were inactive, some previous studies manifested a similar effect of crude water extracts less potent than their corresponding methanolic extracts. [27, 28]

Conclusion

B. variegata has good potential and can be used for the development of an antimalarial drug. Preliminary screening results are sufficiently interesting for further phytochemical investigations to identify the chemical compounds responsible for the antimalarial activity and obtain standardized preparations. Bioassay-guided fractionation of the leaves of *B. variegata* extracts is in progress to isolate and identify the active compounds.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests Not applicable

Funding Not applicable

Authors' contributions

JV designed, performed the laboratory work, analyzed data and drafted the manuscript. NS conceptualize as well as supervised the study and revised the manuscript. VB supervised study and revised the manuscript. JS participated in the laboratory work. All authors read and approved the final manuscript.

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Tables

Table 1
Plants with their families, local name, parts of plant used and traditional uses

Botanical name	Family	Local Name	Plant part	Traditional use and References
<i>Ailanthus Excelsa</i> (AE)	Simaroubaceae	Arduso	Leaves	Leaf juice and bark juice use in forests located near Bhubaneswar, Orissa, India. [10, 11, 12]
<i>Bahaunia variegata</i> (BV)	Caesalpiniaceae	Kachnar	Leaves	South Indians used leaf decoction to relieve headaches in malarial fever [13, 14]
<i>Cesalpinia crista</i> (CC)	Caesalpiniaceae	Fever nut	Leaves	In Assam, India, the leaves and seeds are traditionally used to treat malarial fever [10, 15]
<i>Alstonia scholaris</i> (AS)	Apocynaceae	Saptaparni	Leaves	Diarrhea, dysentery, malaria, and snake bites [10, 16]
<i>Leucas aspera</i> (LA)	Lamiaceae	Dronapushpi	Whole plant	The decoction of whole plant of <i>Leucas aspera</i> are used in malarial [17, 18]
<i>Demia extensa</i> (DE)	Apocynaceae	Dudheli	Roots	Antipyretic, expectorant, malarial intermittent fever [16, 11]
<i>Fagonia Arabica</i> (FA)	Zygophyllaceae	Dhamaso	Whole plant	Fever and malarial fever [19]

Table 2
Plant species with herbarium number and crude extract yields.

Plants	Herbarium numbers	Percentage Yield (%)		
		Methanol	Hydro alcohol	Aqueous
AE	SU/DPS/Herb/58	18.8	24	31.2
BV	SU/DPS/Herb/61	13	15	11.6
CC	SU/DPS/Herb/65	18.8	24	24.2
AS	SU/DPS/Herb/32	11.4	14.6	14.2
LA	SU/DPS/Herb/18	8.6	12	12.2
PD	SU/DPS/Herb/63/A	15.2	14.8	11.2
FA	SU/DPS/Herb/63/B	9.2	8.6	8.2

Table 3

Effect of methanol, hydro alcohol and aqueous extracts of selected medicinal plants on *Plasmodium falciparum* lactate dehydrogenase (PfLDH)

Plant species	IC ₅₀ (µg/mL) (MRC- 2)			IC ₅₀ (µg/mL) (RKL - 9)		
	Methanol	Hydro alcohol	Water	Methanol	Hydro alcohol	Water
AE	102.2**	274.2***	> 500*	274***	313.8***	315.7*
BV	24.65***	39.42***	172.1***	28.71***	62.22***	110.2***
CC	103.3***	142.6***	> 500***	142.6***	217.1***	> 500*
AS	78.11***	88.87***	348.5**	88.87***	132.0***	341.8**
LA	-	431.1***	> 500	431***	> 500*	> 500
DE	> 500	154.6**	> 500*	> 1000	478**	> 500
FA	> 1000	-	> 500	-	-	-
Chloroquine	0.00382**			0.0264		
Artemisinin	0.00221***			0.00401***		

IC₅₀: 50% inhibitory concentration, the number of replicates was three, Significance difference control vs treatment * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figures

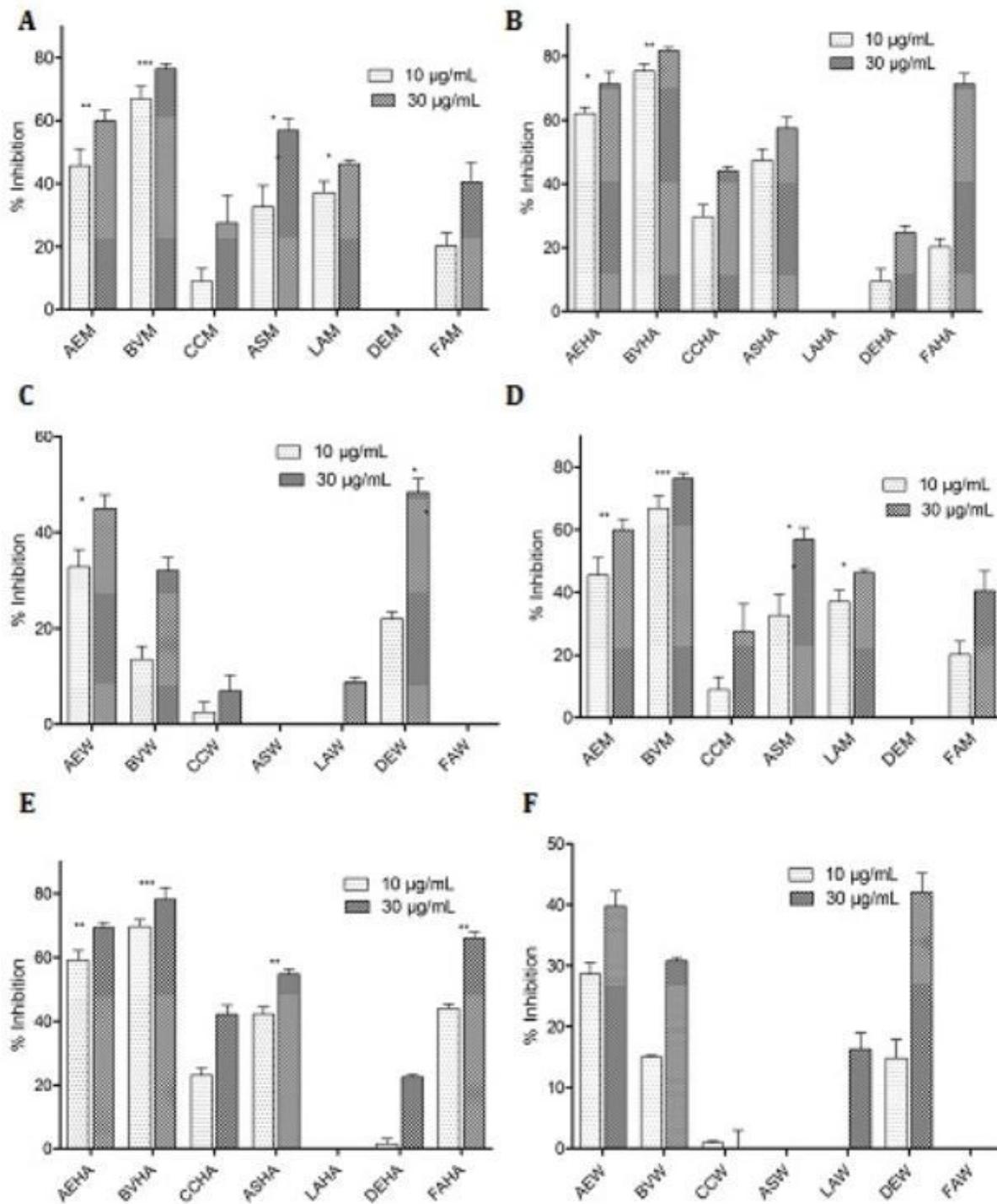


Figure 1

The effect of extracts of plants on schizont maturation of MRC-2 (A, B, C) and RKL-9 (D, E, F) strains. Results are expressed as mean \pm SEM (n = 3). (A-Methanol extract, B- hydroalcohol extract, C- water extract for MRC-2 strain) (D- Methanol extract, E- hydroalcohol extract, F- water extract for RKL-9 strain)