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Pharmacodynamic evaluation for antiplasmodial activity of *Holarrhena antidysentrica* (Kutaja) and *Azadirachta indica* (Neemb) in *Plasmodium berghei* infected mice model

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ABSTRACT

Objective: To investigate *in-vivo* anti-plasmodial activity of aqueous extracts of plants selected based on the symptomology mentioned in Ayurveda. **Methods:** The aqueous extracts of *Holarrhena antidysentrica* (*H. antidysentrica*) (Kutaja) and *Azadirachta indica* (*A. indica*) (Neemb) for their antiplasmodial potential in *Plasmodium berghei* (*P. berghei*) infected mice was assessed using Peters four day suppressive test. Both the extracts were administered at 2 dose levels, full dose (1 000 mg/d) and minimized dose (200 mg/d). 10^6 *P. berghei* infected RBCs were injected on day '0' and treated from day '0' till day '3' post-infection. Tail blood smears were collected, giemsa stained and analyzed. The mice were observed for survival and parasitemia was assessed till 50% of mice in control survived. **Results:** It was observed that the percentage of parasitemia increased gradually in all the groups, with maximum in control group (Day 3-35, Day 9-46.98) and minimum in Chloroquine arm (Day 3-14.06, Day 9-19.92). The percentage of parasitemia was compared using Mann-Whitney *U* test depicting that all test groups exhibited reduction in parasitemia as compared to control (*P*-value<0.002 for all groups). These groups showed similar percentage of survival as Chloroquine. **Conclusions:** The present investigation demonstrated the anti-plasmodial effects of *H. antidysentrica* and *A. indica*, which are two most commonly used medicinal plants in Ayurved for treatment of fever.

1. Introduction

Malaria is a major health problem in India and contributes significantly to the overall malaria burden in Southeast Asia. The National Vector Borne Disease Control Program of India reported ~1.6 million cases and ~1 100 malaria deaths in 2009. Some experts argue that this is a serious underestimation and that the actual number of malaria cases per year is likely between 9 and 50 times greater, with an approximate 13-fold underestimation of malaria-related mortality[1,2]. Complexities in the life cycle of parasite, environmental interactions, high cost of anti-malarial drugs for people living in endemic countries, migration of population between endemic and non-endemic areas and resistance of *Plasmodium falciparum* (*P. falciparum*) to chloroquine, mefloquine and sulfadoxine-pyrimethamine including artemisinin combined therapies, has fuelled the burden of this protozoal infection further more[3-6]. Ever-increasing resistance of malarial parasites to the

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commonly available anti-malarial drugs necessitated the search for new drugs, especially from plants. Traditionally used medicinal plants have played important role in malaria treatment across the globe[7-9]. We investigated the plants selected on the basis of their traditional usage by comparing the disease symptomatology and recommended use from the texts of Ayurveda (Indian System of Medicine) and used them in the forms as used traditionally (water extracts and herb powders) as far as possible. We screened aqueous extracts of two plants *Holarrhena antidysentrica* (*H. antidysentrica*) (Kutaja) and *Azadirachta indica* (*A. indica*) (Neemb) for their antiplasmodial potential in *Plasmodium berghei* (*P. berghei*) infected mice.

2. Materials and methods

2.1. Plant materials

The plants selected *viz. A. indica* (leaves, stem) and *H. antidysentrica* (bark) were collected from Gujarat state of India. The plant species were taxonomically identified and confirmed using morphological and anatomical technique. They were authenticated at the Botanical Survey of India, Pune and voucher specimens were deposited.

2.2. Preparation of samples

Aqueous extracts were prepared from 500 g of fresh raw material of plant parts in a round bottom flask. This mixture was heated in stainless steel extraction vessel under reflux with stirrer for three cycles with water. After each cycle, water was added to replenish the remaining volume. The extracts were dried in a rotatory evaporator under vacuum at temperature not exceeding 35 ℃. The dry extracts of all samples were stored in air-tight sample containers at 4 ℃ until use. For administration to the animals, the aqueous extracts were diluted in distilled water and vortex to get a clear solution.

2.3. Pharmacodynamic evaluation: in vivo antimalarial efficacy testing in P. berghei infected mice

The protocol for animal studies was approved by the Institutional ethical committee of the Tata Institute of Fundamental Research, (TIFR). The protocol was designed on the basis of "Peters four day suppressive test" described by Peters *et al*[10,11]. The lethal strain of *P. berghei* was used for the experiments. In-house bred mycoplasma free male Swiss mice (weighing around 30 g each) were infected by intraperitoneal inoculation of donor mouse blood diluted in acid citrate dextrose (ACD) buffer containing approximately 10^6 *P. berghei* infected RBCs on day '0'. The mice were randomly divided into various groups (n=8 per group) as depicted in Table 1. Starting from day '0' till day '3' post infection, the different groups were given the assigned treatment (or no treatment for control) by oral gavage. The total volume administered was 400 μL. On fourth day, the blood was withdrawn from tail vein and the blood smears were prepared. Blood smears were fixed with methanol and stained with Giemsa stain and the parasites were counted. Parasitemia is the quantitative content of parasites in the blood. Parasitemia was reported as percentage parasitemia after counting 250 RBCs from each slide. Activity of the treatment groups was calculated by the following formula suggested in the standard protocol by Fidock *et al*[12].

Activity = 100-(mean parasitemia of treated group/mean parasitemia of control group)× 100

The animals in all the groups were also monitored for their survival.

Table 1

Summary of the different groups and their treatment regimen used for *in vivo* antimalarial efficacy studies.

Group (n=8)	Infection	Treatment
Full dose of *H. antidysenterica* (HA)	+	1 000 mg/d of *H. antidysenterica*
Minimized dose of *H. antidysenterica* (HA)	+	200 mg/d of *H. antidysenterica*
Full dose of *A. indica* (AI)	+	1 000 mg/d of *A. indica*
Minimized dose of *A. indica* (AI)	+	200 mg/d of *A. indica*
Chloroquine	+	5 mg/kg/d of Chloroquine[13]
Control	+	No treatment

2.4. Statistical analysis

Data was expressed as mean±SD. and parasitemia of the different groups were statistically assessed by unpaired t-test using Graphpad Instat Demo version. Differences were considered significant at $P<0.05$.

1. % Parasitemia of infected mice was calculated:

$$\frac{\text{Number of infected red blood cell}}{\text{Total red blood cell}} \times 100 = \%\ \text{parasitemia}$$

2. % Activity of the treatment groups was calculated by the following formula suggested in the standard protocol by Fidock *et al*[12]

% Activity = 100-(mean parasitemia of treated group/mean parasitemia of control group)× 100

3. Percent suppression of parasitemia was calculated: Parasitemia of control-parasitemia of test/ Parasitemia of control ×100

4. Number of survival days and % survival was calculated.

3. Results

The % parasitemia was calculated till 50% of animals in control survived, which was Day 9. The mean % parasitemia of the mice in each group was as mentioned in Table 2. It was observed that the % parasitemia increased gradually in all the groups, with maximum in control group (Day 3-35, Day 9-46.98) and minimum in Chloroquine arm (Day 3-14.06, Day 9-19.92) (Figure 1).

Table 2

The mean % parasitemia seen in each group was as follows.

Group	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9
Full dose of *H. antidysenterica*	19.58	20.69	26.41	31.68	32.29	33.10
Minimized dose of *H. antidysenterica*	15.54	16.16	16.44	19.23	19.58	20.38
Full dose of *A. indica*	16.10	16.91	23.73	25.87	25.65	26.35
Minimized dose of *A. indica*	16.55	17.17	21.69	24.31	24.52	25.20
Chloroquine	14.06	16.22	17.12	18.04	18.65	19.92
Control	35.00	36.31	37.90	45.46	46.26	46.98

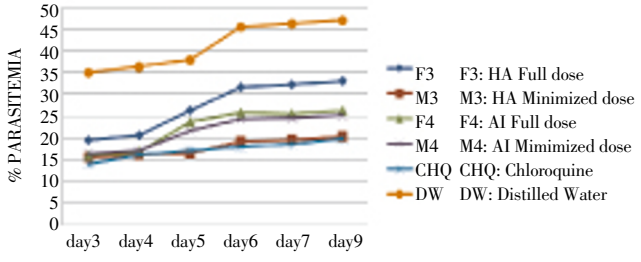

Figure 1. % parasitemia in the groups from day 3 to day 9.

The % parasitemia was compared using Mann-Whitney *U* test depicting that all test groups exhibited reduction in parasitemia as compared to control (*P*-value<0.002 for all groups). While it was observed that the reduction in *H. antidysentrica* minimized dose group and *A. indica* group was comparable to Chloroquine (Figure 2).

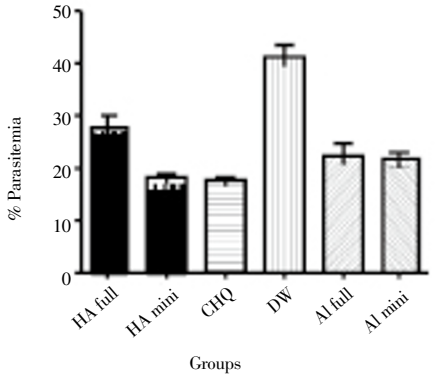

Figure 2. % parasitemia .

It was assessed using Mann-Whitney *U* test, the parasitemia reduction in *H. antidysentrica* minimized dose group and *A. indica* group was comparable to Chloroquine. While all the test groups showed statistically significant reduction as compared to distilled water (*P* value < 0.002 for all groups).

HA full: *H. antidysentrica* full dose Group 1, HA mini: *H. antidysentrica* minimized dose Group 2, AI full: *A. indica* full dose Group 3, AI mini: *A. indica* minimized dose Group 4, CHQ: Chloroquine, DW: Distilled water).

% activity was calculated using the formula as mentioned above (Figure 3). It was observed that minimized dose HA showed comparable activity to Chloroquine. Percent suppression in these 2 groups was similar too. In both these groups 3 animals survied till Day 10, while % survival was highest in minimized dose HA amongst the test groups followed by mimimized dose AI, and full dose AI (Table 3 and 4).

Table 3

In-vivo anti-malarial activity of various formulations in *P. berghei* infected mice (*n*=8).

Treatment group	Average %parasitemia±SD		%activity		No. of survivals
	3rd day	9th day	3rd day	9th day	(*n*=8, t=18 d)
Full dose of *H. antidysenterica*	19.6±2.8	33.1±4.8	44	30	0
Minimized dose of *H. antidysenterica*	15.5±4.1	20.4±3.3	56	57	3
Full dose of *A. indica*	16.1±2.3	26.4±2.3	54	44	2
Minimized dose of *A. indica*	16.5±2.3	25.2±3.0	53	47	2
Chloroquine	14.1±3.3	19.9±2.3	60	58	3
Control	35.0±4.7	47.0±9.6	0	0	0

Table 4

In-vivo anti-malarial activity of plant extracts (*H. antidyesntrica* and *A. indica*) against *P. berghei* infected Swiss albino mice.

Plant extract	Dose	Mean ± SD parasitemia (%)	Percent suppression of parasitemia	% Survival of animal on day 10
H. antidyesntrica	1 000 mg/d	27.3±6.0	34	13
H. antidyesntrica	200 mg/d	17.9±2.1	57	63
A. indica	1 000 mg/d	22.4±4.7	46	38
A. indica	200 mg/d	21.6±3.8	48	50
Chloroquine	5 mg/kg/d	17.3±2.0	58	100
Distilled water	–	41.3±5.5	–	–

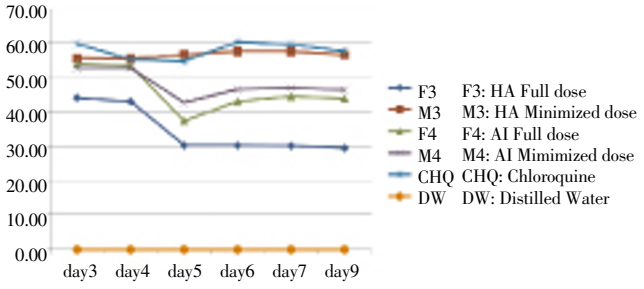

Figure 3. % activity in all the treatment groups compared to the controls.

The survival analysis was performed using ANOVA to compare the 2 dose levels of drugs with chloroquine and control group. Low dose was found to be more efficacious than full dose of test sample, when compared with control (*P*-value <0.000 1) (Figure 4).

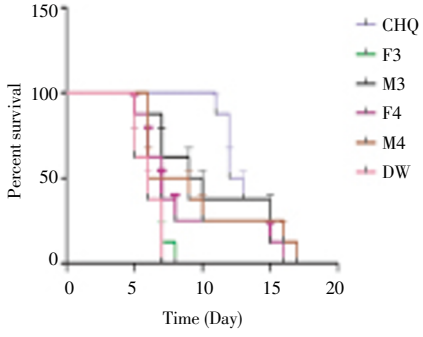

Figure 4. Survival proportions of all the groups.

4. Discussion

In this study, we have found that the both aqueous extracts of *H. antidysentrica* and *A. indica* demonstrated the anti plasmodial property. The parasitemia was reduced in both the dose ranges for these extracts as compared to control (*P* value<0.002). Although *H. antidysentrica* minimized dose and *A. indica* group were comparable to Chloroquine for lowering parasitemia and for survival. *A. indica* minimized dose showed slightly better percent suppression of parasitemia and % survival than its full dose. The present investigation thus demonstrated the anti-plasmodial effects of *H. antidysentrica* and *A. indica* which are two most commonly used medicinal plants in Ayurved for treatment of fever.

The interest in screening of compounds of natural origin or of ethno pharmacological background to develop antimalarial therapy has increased in last few years. Many plants compounds and extracts used traditionally have exhibited promising anti-plasmodial activity. In present study we used 1 000 mg/d and 200 mg/d dose calculated for weight of mice. We used aqueous extracts and water as a solvent to mimic the traditional use of these plant compounds. Percent parasitemia of infected mice and percent activity of the treatment groups were calculated as per the formula mentioned by Fidock *et al*[12]. Percent suppression of parasitemia was assessed using the formula by Obih *et al*[14]. Statistical significant reduction in percent parasitemia of all the test groups was found when compared with the control using Mann Whitney *U* test (*P* value<0.002). The reduction was comparable to Chloroquine group for H.antidysentrica minimized dose group and *A. indica* full dose. The numbers of survival till day 18 were observed in *H. antidysentrica* minimized dose group, *A. indica* full and minimized dose group and Chloroquine indicating the effect of treatment groups on the survival of infected mice. *H. antidysentrica* minimized dose exhibited 57% suppression of parasitemia followed by *A. indica* minimized dose (48%) and *A. indica* full dose (46%) in the treatment groups. The survival was 63% in *H. antidysentrica* minimized dose followed by the *A. indica* minimized dose (50%). It can be noted that both the minimized doses exhibited better parasitemia suppression and survival as compared to full dose. Recently a study published by Verma *et al* screened the 10, 20 & 30 mg/kg of chloroform extract of the *H. antidysentrica* in *P. berghei* infected mice, which demonstrated 73.2% parasite inhibition at 30 mg/kg[15]. There are very few studies for *H. antidysentrica* including the one mentioned above published recently. *A. indica* fractions have shown activities superior to chloroquine *in-vitro*[16,17]. Yet there were very few studies with aqueous extracts of *A. indica* and its activity in a mouse model. Many other Indian medicinal plants have been examined *in-vitro* against *P. falciparum*, of which ethanol extracts *Holarrhena pubescens*, *Pongamia pinnata*, *Plumbago zeylanica* have exhibited IC_{50} of 28 μg/mL, 25 μg/mL and 17 μg/mL[18]. The IC_{50} of various solvent extracts of *Carica papaya*, *Ocimum sanctum*, *Adhatoda vasica*, *etc* indicated anti-plasmodial activity of the traditionally used

medicinal plants[19]. Ellagic acid which is an important chemical constituent of *Punica granatum*, exhibited high *in-vitro* and *in-vivo* antiplasmodial properties and synergistic activity with chloroquine, atovaquone, mefloquine and artesunate[20].

Other traditional medicinal plants from Kenya have also shown low IC_{50} values, high percent suppression and low cytotoxicity, thus indicating potential source for anti-plasmodial agents[21]. *A. indica* along with other Sudanese medicinal plants exhibited highly potent antimalarial property[22]. Thus it is evident that traditionally used medicinal plants have potential and must be explored for novel drug candidate.

Conflict of interest statement

We declare that we have no conflict of interest.

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