Original Article

Antimalarial potential of homeopathic medicines against schizont maturation of *Plasmodium berghei* in short-term *in vitro* culture

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ABSTRACT

In vitro assessment of the susceptibility of *Plasmodium* to antimalarial drugs represents a major research breakthrough that paved the way for the understanding of the parasite, and rapid screening of the effectiveness of antimalarial drugs. In the present study, a preliminary screening of the antiplasmodial activity of the mother tincture (ϕ) and various potencies (6 cH, 30 cH, 200 cH) of homeopathic medicines *China officinalis*, *Chelidonium majus* and *Arsenicum album* was performed by means of the *in vitro* schizont maturation inhibition assay. Significant reduction of the growth of intra-erythrocyte stages of *P. berghei* was observed with decreasing dilutions of ϕ and the various potencies of *Chin*, *Chel* and *Ars* with dose-dependent effect. Maximum schizont maturation inhibition (80%) was observed with *Chin* ϕ (1:1), *Chin* 30 cH (1:1, 1:2) and *Chel* 30 cH (1:1). The standard drug chloroquine (CQ) at 10-µM concentration exhibited 95.4 ± 1.6 % inhibition of schizont maturation. *Ars* 30 (1:1) also exhibited strong antiplasmodial efficacy, with 75.5 ± 2.6 % of schizont inhibition. The presence of free merozoites with *Ars* 200 cH and weak schizont inhibition activity (40-45%) points to the ability of the parasite to survive in the given drug pressure.

Keywords: Homeopathy; *Plasmodium berghei: China officinalis*; *Chelidonium majus; Arsenicum album*

Introduction

Research on malaria has benefited considerably from technical advances in the culture systems for *Plasmodium* [1]. Continuous *in vitro* cultivation of human malaria parasite *P. falciparum* was first reported by Trager and Jenson in 1976. This paved the way for the development of microtiter plate assay for the determination of antiplasmodial activity, in which the parasite growth is assessed by incubating (21 h) culture in RPMI-1640 medium supplemented with various antibiotics [2]. After the establishment of continuous *in vitro* culture of *P. falciparum,* studies on invasion inhibition studies were conducted with different species of the malaria parasite. Ladda *et al* gave a detailed fine structural account of the process of erythrocyte invasion by merozoites of *P. berghei yoelii* and *P. gallinaecum* [3]. McNally *et al* described *in vitro* erythrocyte invasion assays for two species of rodent malaria, namely *P. berghei* and *P. chabaudi*. They demonstrated an *in vitro* preference for the reticulocytes for invasion by both species [4].

Short-term invasion inhibition assays might be used to assess the susceptibility to antimalarial drugs. *In vitro* studies were also performed to determine the resistance of *P. falciparum* to standard antimalarial drugs chloroquine, quinine, amodiaquine, halofantrine, mefloquine, cycloguanil and pyrimethamine [5].

The present study aimed to study *in vitro* the inhibition of *P. berghei* schizont maturation in the presence of various homeopathic drugs, namely *China officinalis*, *Chelidonium majus,* and *Arsenicum album*. According to Bellavite *et al*, as high dilutions (HD) of homeopathic preparations are able to evoke a specific and global secondary healing reaction, they might represent potentially effective therapeutic agents [6].

Also the first *in vitro* study on HD ever published in a mainstream science journal supported the effects of homeopathic solutions *in vitro*. Those authors suggested that extremely diluted solutions of antiserum against human IgE were able to induce basophil degranulation *in vitro* [7]. *In vitro* research using HD was also conducted with molecular or cellular systems. This approach reduces the complexity of the models, and allows for a higher degree of standardization of the drugs compared to clinical research, and might eventually provide model systems to reveal the mechanism of action of HD, traditionally considered to be devoid of pharmacologically active molecules [8]. Extensive *in vitro* studies were performed by a team of researchers at the University of Utrecht using cultured mammal cells, primarily to understand the mechanism of action of homeopathic formulations [9].

Materials and methods

Maintenance of the parasite strain

White Swiss mice *Mus musculus* strain BALB/c, weighing 22-26 g, aged 4-6 weeks old, of either gender, obtained at Central animal house, Panjab University, Chandigarh, India, were used for parasite maintenance. The mice were maintained on a standard pellet diet and water *ad libitum*. The strain of *P. berghei* (NK-65) was maintained by means of intraperitoneal inoculation of 1 x 10^6 infected red blood cells (RBCs) to naïve mice [10]. The treatment of mice followed the guidelines of the animal ethics committee (Reg. No. 45/1999/CPCSEA), Panjab University, Chandigarh. The parasitemia was checked by preparing Giemsa-stained thin blood smears on slides following incision of the tail vein of the infected mice**.**

Experimental drugs

Homeopathic mother tincture (ϕ) and different potencies (6 cH, 30 cH and 200 cH) of *China officinalis*, *Chelidonium majus* and *Arsenicum album* manufactured by Dr. Reckeweg and Co. GmbH D.64625, Bensheim, Germany, were used in the present study.

Chloroquine (CQ - chloroquine phosphate suspension containing 50 mg of base, Lariago®) manufactured in India, was used as standard positive control. The required concentration of 10 µM was prepared according to the method described by the World Health Organization (WHO) [11].

In vitro schizont inhibition assay

Short-term *in vitro* culture of *P. berghei* blood stages was performed according to modified Trager and Jensen method [2]. The *in vitro* antimalarial efficacy of the homeopathic medicines was determined by means of schizont inhibition assay [11].

Short-term *in vitro* culture

RPMI-1640 medium (Gibco) supplemented with 0.06% (w/v) HEPES, 5% (w/v) sodium bicarbonate; antibiotics – gentamycin (50 µg/ml), penicillin (100 µl/ml) and streptomycin (100 µg/ml) was used as culture medium (pH 7.4). Ten percent (v/v) inactivated fetal calf serum (FCS) was added to the incomplete medium to prepare the complete medium.

Preparation of normal and *P. berghei*-infected erythrocytes

Blood from normal mice and *P. berghei* infected mice was collected aseptically in citrate saline and centrifuged at 1,000 g for 10 min for pellet separation. The pellet was washed with the incomplete medium. Parasite pellet from infected blood containing rings at lower layer was aspirated. Infected and normal erythrocytes were mixed in a proportion to achieve a parasitemia level of 2-4% at 0 h.

***In vitro* antiplasmodial activity of the homeopathic drugs**

The antiplasmodial activity of various dilutions (1:1, 1:2 and 1:4) of the mother tincture and different potencies of the homeopathic drugs was checked by assessing the inhibition of schizonts following incubation for 21 h (WHO, 2001). One ml of the complete medium contained 50 µl of homeopathic drugs *Chin*, *Chel*, or *Ars* (ϕ, 6 cH, 30 cH, and 200 cH) diluted in different ratios. Each drug was checked in duplicate in a 24-well microtiter plate. After shaking gently the titer plate, 0 h smears were prepared, and the culture plate was incubated at 37 °C in a candle jar (5% CO_2, 17% O_2, 78% N_2) according to Trager and Jensen's (1976) method. After 21 h of incubation, the smears from each well were prepared, fixed in methanol, and stained with Giemsa dye. Inhibition of schizont development by comparison to the control wells was determined by following equation: 100 - [A/B ×100], where A is the average number of schizonts in the drug treated well, and B is the average number of schizonts in the infected control wells.

The experiment was repeated three times to validate the results. The data are expreesed as mean and standard deviation (SD). The statistical significance and intergroup difference in schizont maturation inhibition by the various drugs were assessed by paired Student's t-test. Comparison was performedbetween the test and control groups of mice with p value < 0.05 as statistically significant.

Results

***In vitro* schizont maturation inhibition assay**

The short-term *in vitro* culture of *P. berghei* was tested as to the antiplasmodial efficacy of the homeopathic medicines. Three different dilutions (1:1, 1:2 and 1:4) of each drug were used to check the drug-dependent effect on intra-erythrocyte development of *P. berghei in vitro*.

The lower layer of hematocrit consisting of rings and trophozoites were used for initiating culture. 2.8% infection was observed at 0 h (Fig. 1A). After 21 h of incubation at 37°C, the culture was terminated and development of schizonts was checked. There was almost fivefold increase in the parasitaemia (14.7%) after 21 h of incubation (Fig. 1B and C).

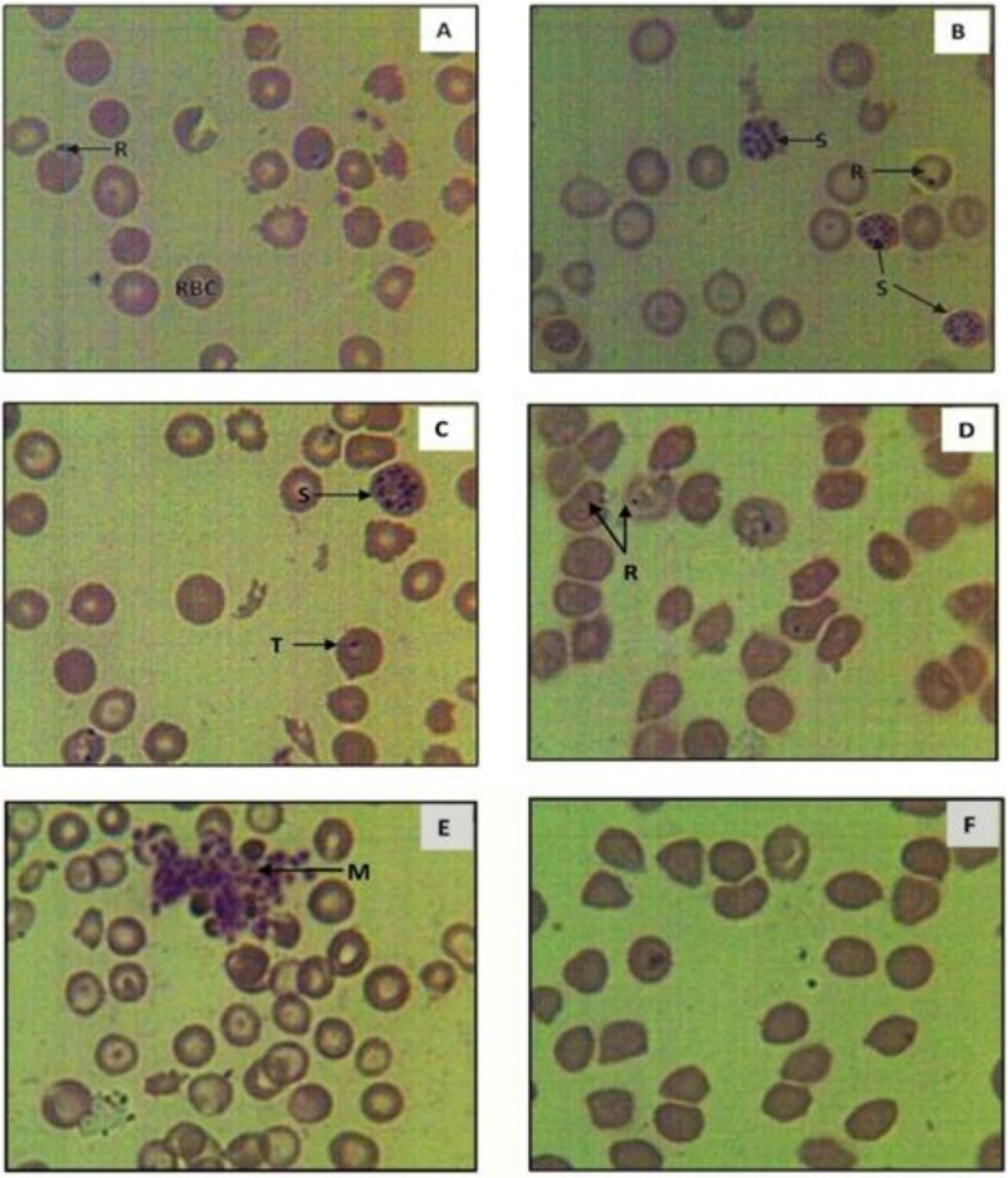

Figure 1: Giemsa-stained blood smears (100×) of *P. berghei in vitro* culture at 0 h (A), 21 h (B, C) along with *Chin* 200 cH (D), *Ars* 200 cH showing free merozoites (E), and CQ 10μM (F) after 21 h of incubation. RBC-normal red blood cell, R - ring, T - trophozoite, S - schizont, M - free merozoites.

The mother tincture and different potencies of *Chin*, *Chel*, and *Ars* inhibited the development of intra-erythrocyte stages of *P. berghei in vitro* in a dose dependent manner (Fig. 2, 4, 6). Decrease in schizont maturation inhibition was observed with increase in the dilution of drug (Fig. 3). Maximum schizont maturation inhibition (80.2 ± 2.1 %) was observed in the presence of *Chin* ϕ. *Chin* 30 cH exhibited 80% inhibition of schizont maturation after 21 h of incubation. *Chin* 200 cH exhibited (50.0 ± 0.8)% of schizont maturation inhibition.

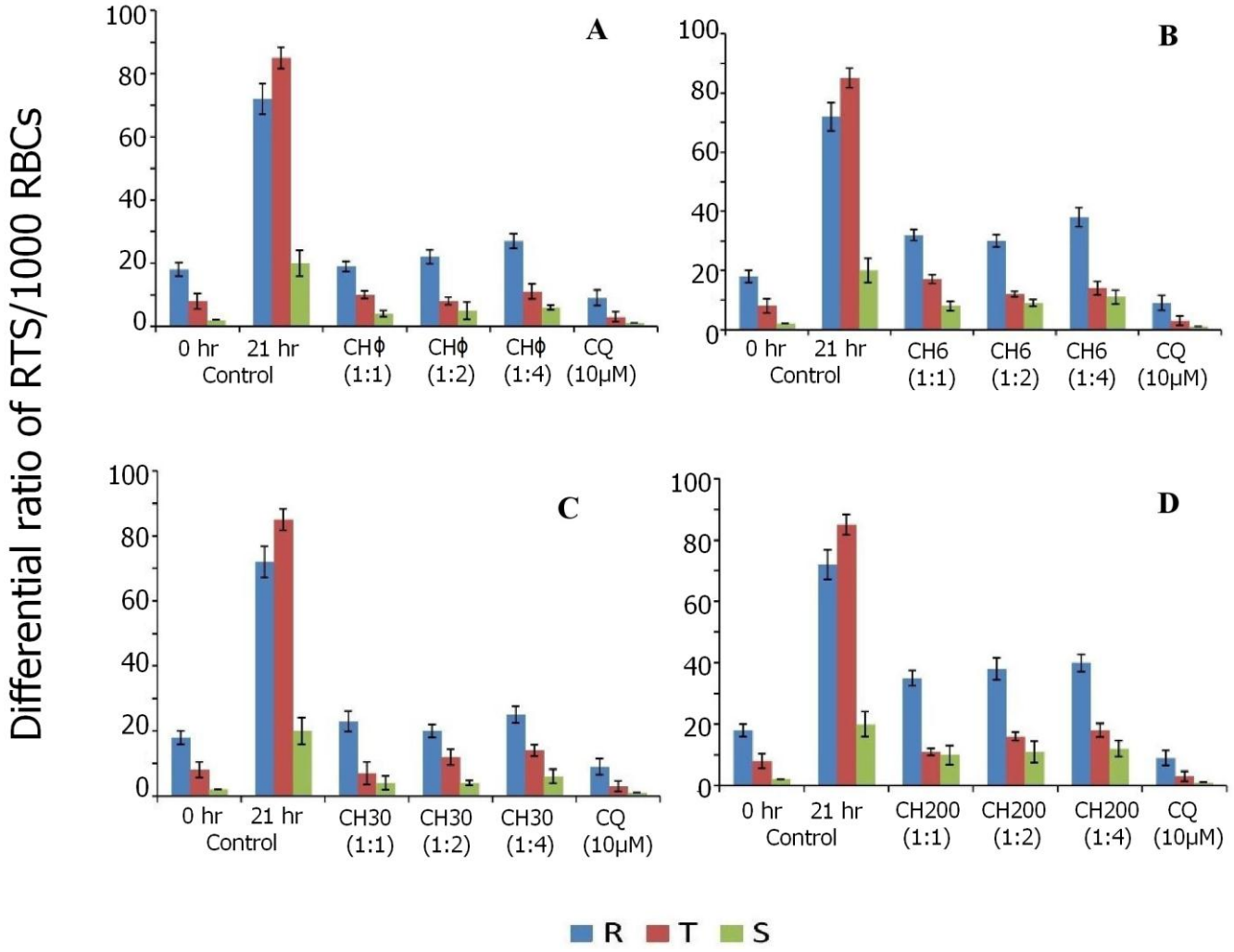

Figure 2: Histogram showing dose-dependent effect on intra-erythrocyte stages of *P. berghei in vitro* after 21 h of incubation in the presence of *Chin* ϕ (A) and its various potencies: *Chin* 6 cH (B), *Chin* 30 cH (C) and *Chin* 200 cH (D) along with positive control CQ (10μM). R - rings, T - trophozoites, S - schizonts. Data are expressed as the mean ± SD of three separate experiments.

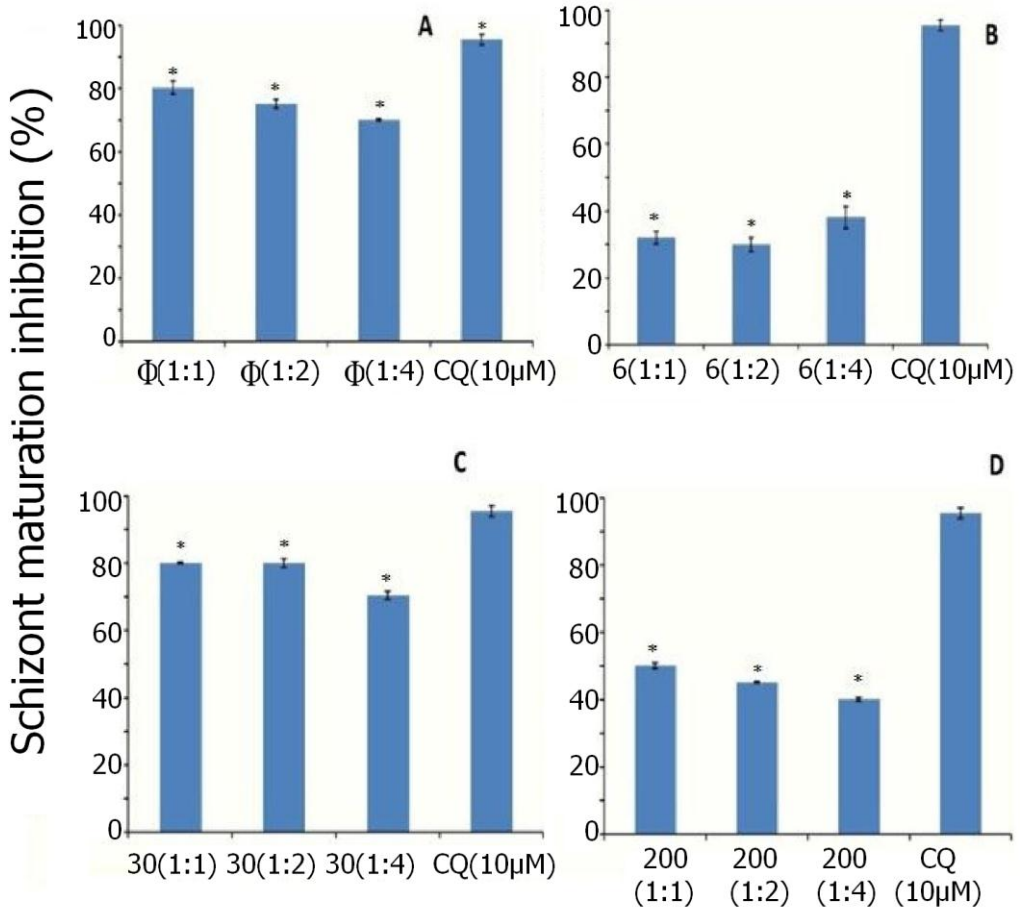

Figure 3: Histogram showing schizont maturation inhibition of *P. berghei in vitro* after 21 h of incubation in the presence of *Chin* ϕ (A) and its various potencies: *Chin* 6 cH (B), *Chin* 30 cH (C) and *Chin* 200 cH (D) along with positive control CQ (10μM). Data are expressed as the mean ± SD of three separate experiments. $p<0.05$ in comparison to positive control CQ (10μM) is shown as * (statistically significant).

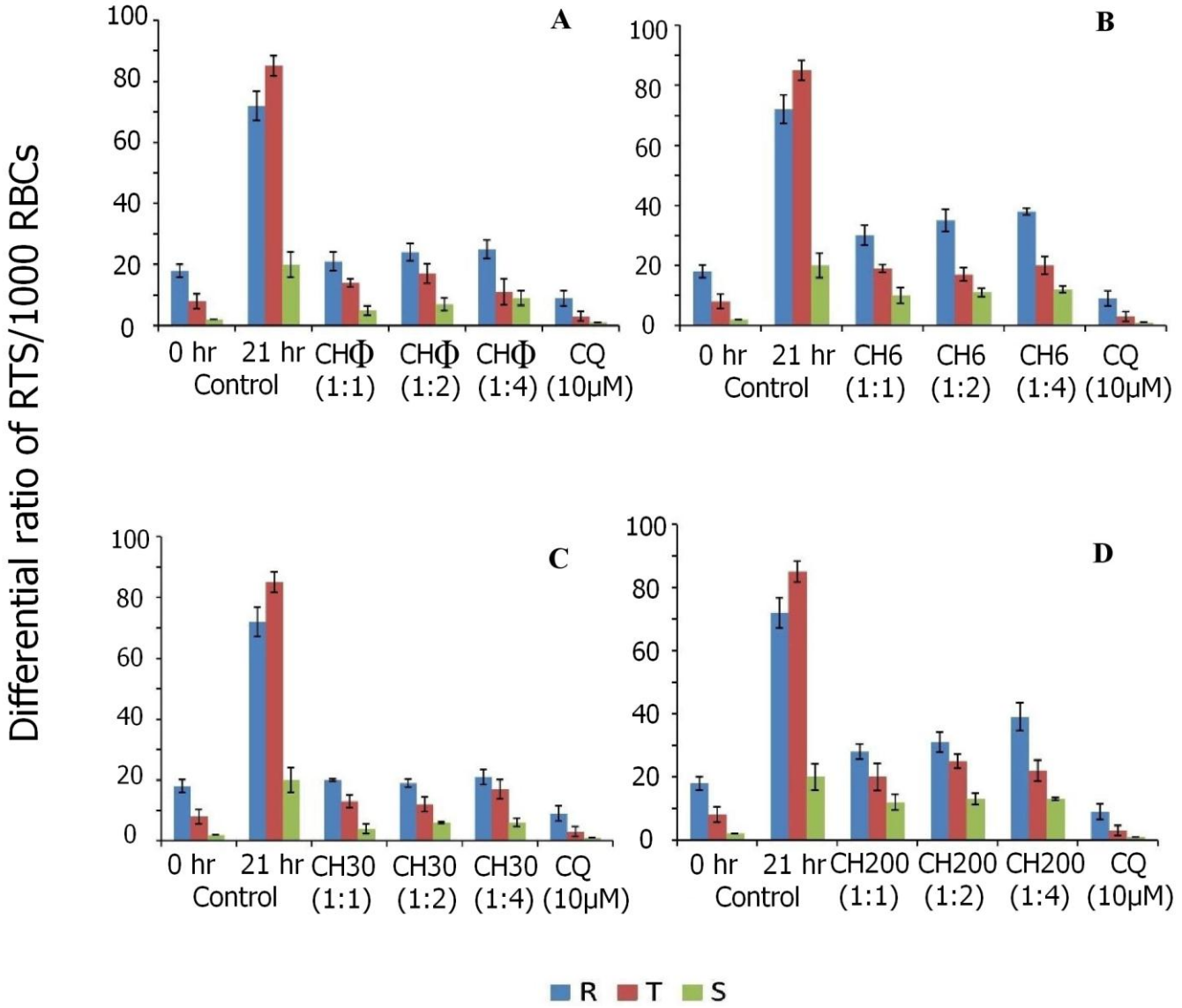

Figure 4: Histogram showing dose-dependent effect on intra-erythrocyte stages of *P. berghei in vitro* after 21 h of incubation in the presence of *Chel* ϕ (A) and its various potencies: *Chel* 6 (B), *Chel* 30 cH (C) and *Chel* 200 cH (D) along with positive control CQ (10μM). R - rings, T - trophozoites, S - schizonts. Data are expressed as the mean ± SD of three separate experiments.

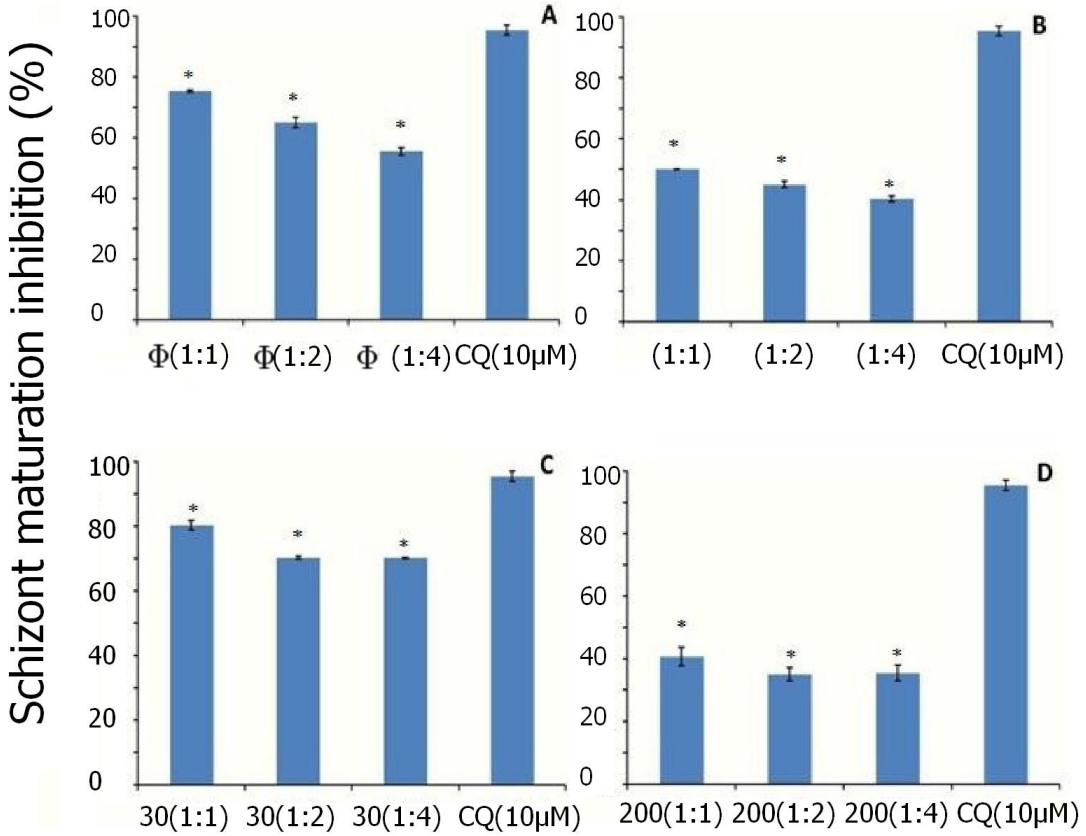

Figure 5: Histogram showing schizont maturation inhibition of *P. berghei in vitro* after 21h of incubation in the presence of *Chel* ϕ (A) and its various potencies: *Chel* 6 cH (B), *Chel* 30 cH (C), *Chel* 200 cH (D) along with positive control CQ (10 μM). Data are expressed as the mean ± SD of three separate experiments. $p<0.05$ in comparison to positive control CQ (10 μM) is shown as * (statistically significant).

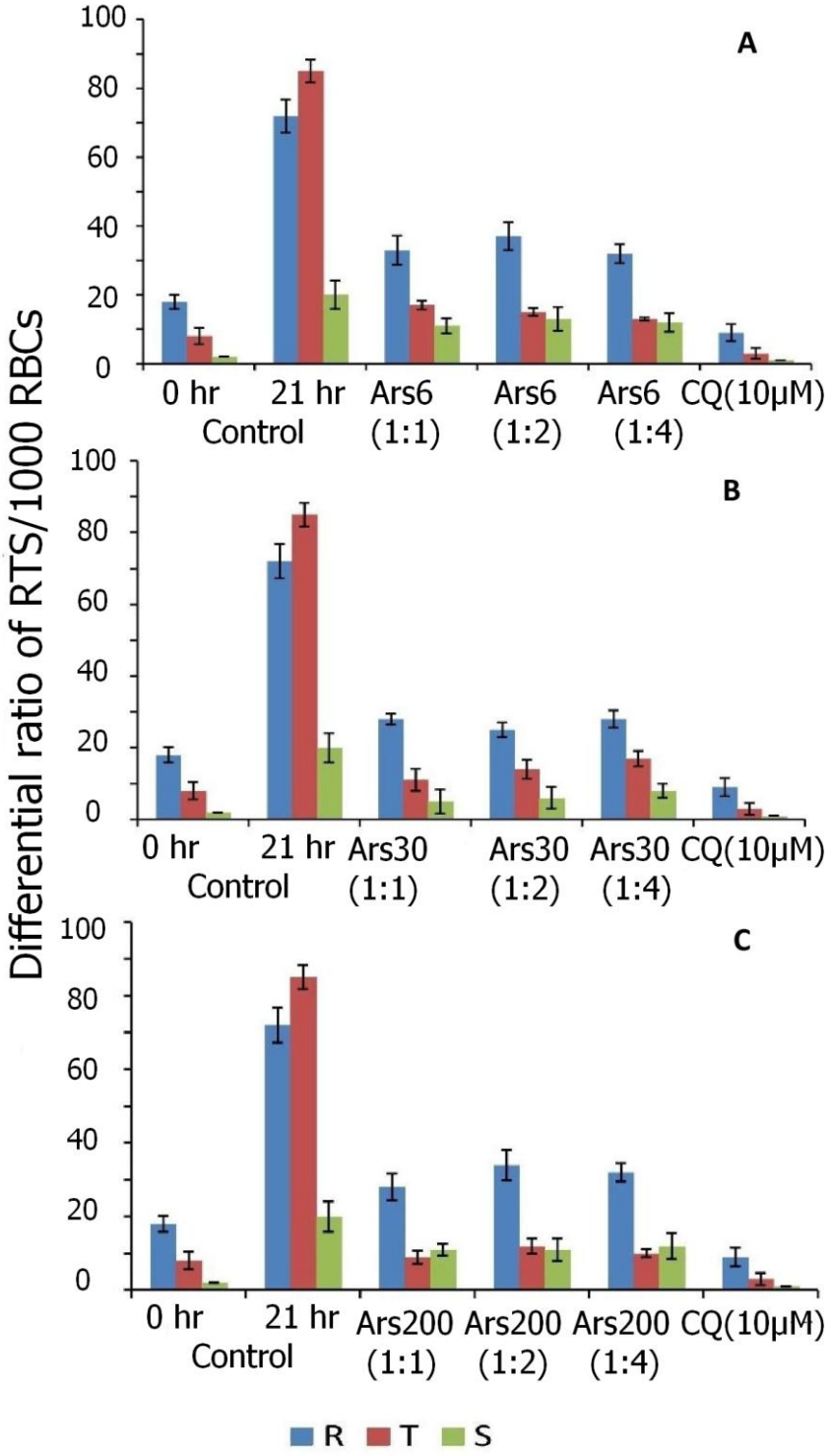

Figure 6: Histogram showing dose-dependent effect on intra-erythrocyte stages of *P. berghei in vitro* after 21 h of incubation in the presence of *Ars* 6 cH (A), *Ars* 30 cH (B) and *Ars* 200 cH (C) along with positive control CQ (10μM). R - rings, T - trophozoites, S - schizonts. Data are expressed as the mean ± SD of three separate experiments. R T S

A total of 80.2 ± 1.5 % inhibition of schizont maturation was observed in the presence of *Chel* 30 cH. As the dilution of the drug was increased, decline in the schizont maturation inhibition was observed in ϕ and the various potencies (Fig. 5). Chloroquine (CQ) 10 µM exhibited 95.4 ± 1.6 % inhibition of schizont maturation after 21 h of incubation *in vitro*.

The maximum schizont maturation inhibition of *Arsenicum album* was observed with *Ars* 30 cH (75.5 ± 2.6 %). *Ars* 6 cH and *Ars* 200 cH exhibited about 45% inhibition of schizont maturation (Fig. 7). Many live free merozoites were also observed outside the RBCs in the smears of *Ars* 200 cH-treated wells (Fig. 1E). The maximum schizont maturation inhibition (95.4 ± 1.6 %) was observed in the positive control CQ (10 µM) (Fig. 1F).

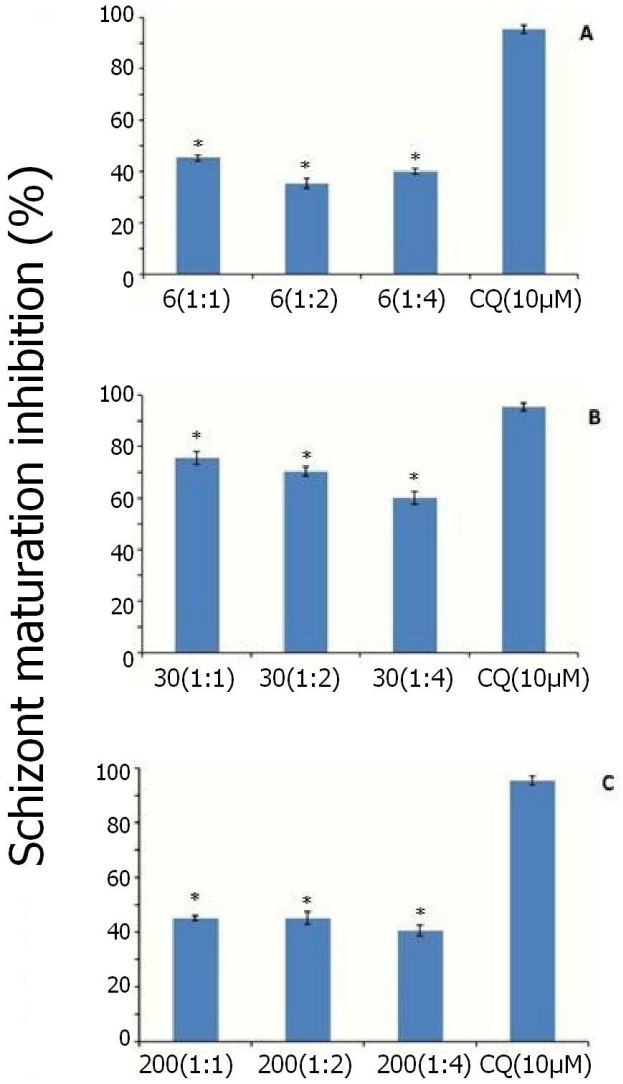

Figure 7: Histogram showing schizont maturation inhibition of *P. berghei in vitro* after 21 h of incubation in the presence of *Ars* 6 cH (A), *Ars* 30 cH (B) and *Ars* 200 cH (C) along with positive control CQ (10 µM). Data are expressed as the mean ± SD of three separate experiments. $p<0.05$ in comparison to positive control CQ (10 µM) is shown as * (statistically significant).

Discussion

The parasite grows and multiplies in the host's red blood cells, where it modifies the membrane permeability and the cytosolic composition. Erythrocytes serve as a new model to assess drug susceptibility in rodent malaria [12]. The development of techniques for *in vitro* cultivation of *Plasmodium* by Trager & Jensen led to the development of an assay system of drug susceptibility useful in both field and laboratory conditions.

The experiments described in the present study provide evidence and detailed methods for performing erythrocyte invasion/inhibition assays with *P. berghei* in short-term *in vitro* culture. *In vitro* antimalarial drug testing is an essential tool for the objective evaluation of the efficacy of antimalarial drugs in concentration-dependent effect on parasite grow.

Cultivation of human and non-human *Plasmodium* species *in vitro* represents a major research breakthrough that paved the way for a better understanding of the parasite, and rapid screening of the effectiveness of antimalarial drugs. *In vitro* assays are able to test several compounds quickly, at low cost, and require small amounts of the drugs.

The present study points to significant antiplasmodial efficacy in *Chin*, Chel, and *Ars*, whereby the maximum percentage of schizont maturation inhibition (80%) was exhibited by *Chin* ϕ (1:1), *Chin* 30 cH (1:1), *Chin*, 30 cH (1:2) and *Chel* 30 cH (1:1). The investigated potencies of *Ars* exhibited weak antiplasmodial efficacy compared to *Chin* and *Chel,*, and maximum schizont maturation inhibition of 75% was found with *Ars* 30 (1:1) *in vitro*.

All three investigated homeopathic medicines were found to inhibit *P. berghei* schizont maturation in a dose-dependent manner, and significant reduction of the growth of intra-erythrocyte stages of *P. berghei* was observed with decreasing dilutions of ϕ and the various potencies (6 cH, 30 cH and 200 cH).

The presence of free merozoites in the *Ars*-treated wells points to the ability of the parasite to survive in the given drug pressure. Also Landau *et al* proposed the presence of latent merozoites being able to penetrate into the erythrocytes [13]. Free merozoites in the circulation are believed not to be vulnerable to the action of antimalarial drugs [14].

The standard drug, CQ at 10-μM concentration exhibited 95.4±1.6% inhibition of schizont maturation *in vitro* in the present study. Tasanor et al developed an *in vitro* test system for short-term culture of *P. vivax* to assess the parasite's sensitivity to chloroquine. That test system was evaluated in 200 fresh *P. vivax* isolates in an area with satisfactory clinical-parasitological response to chloroquine. At 30 or 42 h of incubation, 121 isolates (61.5%) showed adequate control of growth and yielded valid sensitivity tests. Complete inhibition of the parasite development occurred within the concentration range of 40–1280 nM [15].

The present study points to the considerable antiplasmodial activity of homeopathic drugs against intra-erythrocyte growth of *P. berghei* in an *in vitro* system. Further investigation is needed to explore the *in vivo* therapeutic efficacy of these medicines, which may support the use of homeopathy in the treatment of malaria.

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Potencial antimalárico de medicamentos homeopáticos contra a maturação de esquizontes de *Plasmodium berghei* em cultura *in vitro*

RESUMO

O estudo *in vitro* da susceptibilidade de *Plasmodium* a drogras antimaláricas representa um grande avanço nas pesquisas, abrindo novas rotas para o entendimento do parasite e da efetividade de drogas antiomaláricas. Nesse trabalho, realizamos um estudo preliminar da atividade antiplasmódica da tintura mãe (ϕ) e várias potências (6 cH, 30 cH, 200 cH) dos medicamentos homeopáticos *China officinalis (Chin)*, *Chelidonium majus (Chel)* e *Arsenicum album (Ars)*, através do estudo *in vitro* da inibição da maturação de esquizontes. Observamos uma redução significativa do crescimento do estágio intra-eritrócito do *P. berghei* conforme a tintura mãe e demais potências de *Chin*, *Chel* e *Ars* foram diluídas, observando-se um efeito dependente da dose. O máximo de inibição na maturação dos esquizontes (80%) foi observado com *Chin* ϕ (1:1), *Chin* 30 cH (1:1, 1:2) and *Chel* 30 cH (1:1). A droga Cloroquina (CQ), usada como controle, em uma concentração de 10μM, exibiu (95.4 ± 1.6) % de inibição. *Ars* 30cH (1:1) também apresentou uma forte eficácia antiplasmódica com (75.5 ± 2.6) % de inibição de esquizontes. A presence de merozoites livres com *Ars* 200 cH e uma fraca atividade inibidora (40-45%) indicam a habilidade do parasita em sobreviver na presença dessa droga.

Palavras-Chaves: Homeopatia; *Plasmodium berghei: China officinalis*; *Chelidonium majus; Arsenicum album*

Potencial antimalárico de medicamentos homeopáticos contra la maduração de esquizontes de *Plasmodium berghei* en cultura *in vitro*

RESUMEN

El estudio *in vitro* de la susceptibilidad del *Plasmodium* a drogras anti-maláricas representa un gran avance en la investigación y la apertura de nuevas rutas para la comprensión del parásito y la eficacia de los fármacos antimaláricos. En este trabajo se realizó un estudio preliminar de la actividad antiplasmodial de la tintura madre (ϕ) y de diversas potencias (6 cH, 30 cH, 200 cH) de los medicamentos *China officinalis* (*Chin*), *Chelidonium majus* (*Chel*) y *Arsenicum album* (*Ars*) mediante el estudio *in vitro* de la inhibición de la maduración de esquizontes. Se observó una reducción significativa en el crecimiento de la etapa intra-eritrocítica de *P. berghei* conforme la tintura madre y otras potencias de Chin Chel e Ars fueron diluídas, con la observación de un efecto dosis dependiente. La máxima inhibición de la maduración de esquizontes (80%) se observó con *Chin* ϕ (1:1), *Chin* 30cH (1:1, 1:2) y *Chel* 30cH (1:1). El contról Cloroquina (CQ), a una concentración de 10 μM, exhibió (95,4 ± 1,6)% de inhibición. Ars 30cH (1:1) también mostró una fuerte eficacia en la inhibición de los esquizontes (75,5 ± 2,6%). La presencia de merozoitos libres con Ars 200 cH y una débil actividad inhibidora (40-45%) indican la capacidad del parásito en sobrevivir en la presencia de esta droga.

Palabras-clave: Homeopatia; *Plasmodium berghei: China officinalis*; *Chelidonium majus; Arsenicum album*

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