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Effect of *Crocus sativus* Stigma (saffron) alone or in combination with chloroquine on chloroquine sensitive strain of *Plasmodium berghei* in mice

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ABSTRACT

Introduction: In malaria treatment protocols, treatment failure or drug resistance of synthesized drugs like alkaloids related to quinine, and aminoquinolines are the main problems now. Therefore, discovering efficient drugs or combination therapy of blood schizonticidal drugs with different mechanisms or different targets in the parasite is a crucial effort to solve this problem. In this study, the effectiveness of *Crocus sativus* Stigma (saffron) individually and in combination with chloroquine, was considered against chloroquine–sensitive strain of *Plasmodium berghei*.

Methods: At the first stage, using 4 day suppressive Peter's test in mice, ED_{50} and survival times of saffron methanol extract, and its aqueous and ethyl acetate fractions and chloroquine on *P. berghei* were calculated. Then, based on the toxicity and survival time results, combination therapy was conducted with the best saffron fraction and chloroquine against the parasite.

Results: The saffron extract, aqueous and ethyl acetate fractions resulted in suppression of parasitemia with ED_{50} values of 587.0 ± 78.7, 323.7 ± 37.2, and 508.7 ± 35.6 mg/kg, respectively. Combination of ethyl acetate fraction with chloroquine, potentiated the antimalarial property and the survived percent of the treated mice on days 7, 14, and 28 significantly more than chloroquine or ethyl acetate fraction alone.

Conclusion: Saffron and its fractions individually can be effective in reducing the parasitemia in mice. The outcome of combination of ethyl acetate fraction with chloroquine on the mice showed synergistic effect on the chloroquine–sensitive strain of parasite.

Implication for health policy/practice/research/medical education:
Saffron and its fractions are effective in reducing the parasitemia in mice. The combination of ethyl acetate fraction with chloroquine has synergistic effect on the chloroquine–sensitive strain of parasite. Hence, the combination therapy might be useful in the treatment of malaria.

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Introduction

In the mid of 19th century, it was supposed that malaria would be eradicated but now the disease is reached to an epidemic problem in the tropical regions in Africa and south of Asia. It is due to the several factors including increasing the drug resistance of malarial parasites, and limitation of use of insecticides because of toxicological problems and consequent failure in controlling anopheline mosquitoes. Malaria is caused by *Plasmodium falciparum*, *P. vivax, P. malariae* or *P. ovale*. Among them *P. falciparum* is reported to have more morbidity because of resistance of plasmodiums to chloroquine or other drugs. *P. vivax* is usually sensitive to treatment and *P. malariae* and *P. ovale* are uncommon (1). Current anti malarial drugs include quinine an alkaloid extracted from the bark of cinchona tree which is frequently used in the prevention and treatment of malaria. Quinimax, quinidine and mefloquine are other alkaloids related to quinine in the treatment or pre-

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vention of malaria (2). Chloroquine, and amodiaquine are another widely used antimalarial drugs with 4-aminoquinolone structure but, drug-resistant was rapidly decreased their effectiveness (2,3). Primaquine is 8-aminoquinolone for treatment of different types of malaria infection with blocking the oxidative metabolism in plasmodia (2). Artemisinin and artesunate are sesquiterpene lactones with a chemically rare peroxide bridge linkage effective against multidrug resistant *P. falciparum* (4).

Despite the administration of drugs, increasing the risks of treatment failure because of developing resistance is the main problem, now. Therefore, discovering new and efficient drugs or combination therapy of 2 or more blood schizonticidal drugs with different mechanisms and different targets in the parasite is a crucial effort to solve this problem (5). Saffron is a spice derived from the styles and stigmas of the flower of *Crocus sativus* L., commonly known as the "za'ferân" in Persian. It is grown from Spain in the west to India in the east but southeastern provinces in Iran including Khorasan region produce approximately most of the world production (6). The stigmas are mostly used as food spice and colorant. There are also some reports on the effectiveness of saffron for the treatment of malaria (*Tab-e- se-yek* in Persian) in the Islamic medical manuscripts (7). Saffron contains carotenoids, including zeaxanthin, lycopene, and α- and β-carotenes (8). Crocin is a carotenoid that may comprise up to 10% of dried saffron. Picrocrocin and safranal has insecticidal and pesticidal properties and may comprise to more than 4% of saffron's mass (8). In a recent research crocin derived from saffron and safranal semi-synthetic derivatives showed in vitro anti-malarial properties (9). Therefore, saffron was selected alone or in combination with chloroquine in this study for evaluation of its anti-malarial properties in *P. berghei* infected mice as an experimental in viv*o* model.

Materials and Methods

Plant material

Saffron, the dried stigmas of the *Crocus sativus* flowers, was obtained from Global international saffron company, Torbat-e-Heydarieh, Khorasan, Iran and identified at the Pharmacognosy Department, School of Pharmacy, Isfahan University of Medical Sciences. The plant material was milled into a fine powder (100 g) and then macerated with ethanol (600 mL × 3), at room temperature for three days. The extract was evaporated in a vacuum system at 40°C (Rotary evaporator, Heidolph Co. Germany) to a dark red residue which was subjected to liquid/liquid partitioning between water and ethyl acetate. Aqueous and ethyl acetate fractions were evaporated to remove solvent and kept in refrigerator in -20°C before use.

Animals

Male albino sourian mice, weighing between 23 to 27 g supplied from Pasteur Institute of Iran, were randomly assigned to treatment and control groups with 7 mice in each group. The mice were kept at room temperature in accordance with the internationally accepted principles for laboratory animal use and care, and were given ad libitum access to food and water.

Parasites

Chloroquine-sensitive *P. berghei* NICD strain from Haffkine Institute of India was used in the study. Two weeks previous to the tests the parasites (stored in nitrogen liquid) were rethawed and maintained by blood passage in saurian mice, intra-peritoneal. The donor mice with 10% parasitaemia were then sacrificed and blood was collected and diluted with normal saline into heparinized falcon tubes (5×10^6/mL infected erythrocytes). Treatment mice were inoculated with 10^6 parasitized erythrocytes in 0.2 ml dilution, intra peritoneal.

Antimalarial in vivo assay

The anti-malarial study requires in vitro methods or in vivo test models. The most common in vivo test utilize *P. berghei* in a 4 day suppressive test in mice (Peter's test). Different dose levels (350, 700, and 1000 mg/kg) of saffron total extract, aqueous and ethyl acetate fractions and chloroquine (20 mg/kg) were administered to groups of 5 mice in each group in addition to 5 mice uninfected mice as negative control and 5 infected but treated with physiological saline + 2% tween 80 as placebo were prepared. Two hours after parasite inoculations in day zero, plant extracts and chloroquine were injected into the infected mice and repeated once daily for 4 consecutive days. On fifth day a thin blood smear of the tail blood of each of the mice was prepared on microscope slides, fixed with absolute methanol, and stained with 10% Giemsa stain in distilled water. Percentage of parasitaemia suppression was determined by counting parasites against 200 erythrocytes on days 5, 7, 14 and 28 for Peter's test using the following formula (10).

$$\%\,\text{suppression} = \frac{\text{Mean parasitemia of placebo} - \text{mean parasitemia of treated group}}{\text{Mean parasitemia of placebo}}$$

If any animals died before the end of the fourth day, the death was considered for the extract toxicity (1). Combination therapy was then conducted with three treatment groups 1, 2 and 3 treated with best fraction of saffron extract, chloroquine (20 mg/kg) and a combination of these two, in addition to negative control (nor infected , nor receiving any of the agents) and placebo. Interaction was evaluated as additive, synergism or antagonism, respectively. The treated mice were followed up in both screening and combination method for 28 days after injection (on days 5, 7, 14, and 28), and survival time of the mice was recorded in each test group (10-12).

Ethics

All the animals were handled in accordance with the internationally accepted principles and guides for the care and use of laboratory animals in 2010 (11), and the Ethics Committee of Isfahan University of Medical Sciences.

Statistical analysis

The ED_{50} value (drug concentration causing 50% of maximum suppression) of the samples were calculated based on semi-log graphs. Values are given as mean ± standard

error of mean (SEM). Statistical analysis was performed using Student's *t* test and/or one-way analysis of variance (ANOVA) as appropriate and Bonferroni post hoc test to identify the differences between treated groups and control.

Results

General mean ± SD for all study samples for age and weight on day zero was 4 weeks old and 27.1 ± 3.4 g, respectively. There were no statistically significant differences between groups in terms of basic characteristics.

The data were analyzed with 2 factors related to the parasitemia suppression and survival time.

In between group analysis, there was a significant difference between the mean of placebo and total extract and ethyl acetate fraction at concentrations of 700 and 1050 mg/kg on day 4 (24 hours after the last dose), $P < 0.001$. Aqueous extract in all the concentrations (350, and 1050 mg/kg) showed parasitemia suppression significantly more than placebo ($P < 0.001$; Figure 1).

The saffron extract, aqueous and ethyl acetate fractions resulted in suppression of parasitemia with ED_{50} values of 587.0 ± 78.7, 323.7 ± 37.2, and 508.7 ± 35.6 mg/kg, respectively. It means that aqueous fraction with lower ED_{50} is more effective in decreasing the parasitemia than ethyl acetate and total extract. However, none of the observed activities of the extracts and fractions was comparable to that of chloroquine ($P < 0.05$).

The mice were followed up for 28 days and ANOVA results showed that mean survival time of the treated mice with chloroquine 20 mg/kg (23.6 days) and ethyl acetate 700 mg/kg (17.8 days) were significantly ($P < 0.05$) more than placebo (10.8 days) while total extract and aqueous fraction treated groups did not show significant differences with placebo. It means that ethyl acetate fraction (700 mg/kg) has longer survival time than other saffron derived treated groups.

Considering the toxicity of total extract, ethyl acetate and aqueous fraction in various concentrations, aqueous fraction (1050 mg/kg) showed more toxicity with two mice died and ethyl acetate fraction (700 mg/kg) exhibited less toxicity with no death in mice under test before the end of the fourth day.

Combination therapy was then conducted with three treatment groups 1, 2 and 3 treated with ethyl acetate fraction of saffron extract (700 mg/kg), chloroquine (20 mg/kg) and a combination of these 2, in addition to negative control and placebo (Figure 2).

There was a significant difference between the parasitemia suppression of ethyl acetate fraction, chloroquine and combination of these 2 with placebo on day 4 ($P < 0.001$) but there was no statistically significant difference between combination group and chloroquine alone ($P > 0.05$).

ANOVA results showed that survived percent of the treated mice with chloroquine + ethyl acetate fraction on days 7, 14, and 28 were significantly ($P < 0.05$) more than chloroquine or ethyl acetate fraction alone. It means that survival time interaction between ethyl acetate fraction and chloroquine against the *P. berghei* showed a synergism pattern (Table 1).

Discussion

The saffron total extract, aqueous and ethyl acetate fractions significantly resulted in suppression of parasitemia in mice infected with *P. berghei*. However, their effectiveness against *P. berghei* parasitemia was not comparable with chloroquine. Combination therapy of chloroquine (20 mg/kg) with saffron ethyl acetate fraction (700 mg/kg) significantly prolonged survival time against the *P. berghei* parasitemia in comparison with chloroquine or ethyl acetate fraction alone.

Investigation on the antimalarial effects of medicinal herbs alone or in combination with current antimalarial drugs opened a new field of research against plasmodium infections.

Motevalli Haghi et al (12) examined the effectiveness of *Peganum harmala* on *P. berghei*. with considerable effect in 100 mg/kg dosage. Nahrevanian and colleagues reported antimalarial activity of *Artemisia khorassanica* and *Artemisia sieberi* against *P. berghei* in mice (13,14). Different parts of plants of the Simaroubaceae family are used traditionally in the treatment of malaria. These plants contain quassinoid alkaloids like simaroubolide compound that are responsible for this activity (15). Stem bark of Alstonia

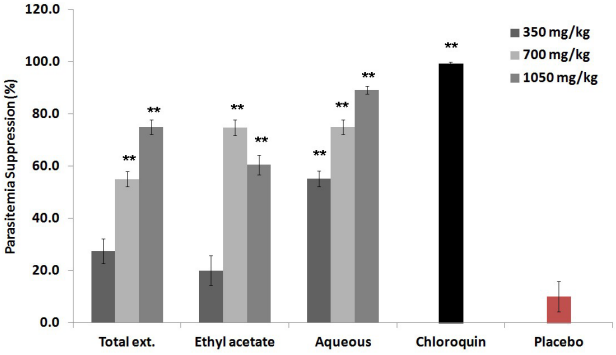

Figure 1. Parasitemia suppression (mean ± SEM) of infected mice treated with crude extract, ethyl acetate and aqueous fractions of saffron on day 4 (1 day after cessation of treatment) in Peter's antimalarial test.

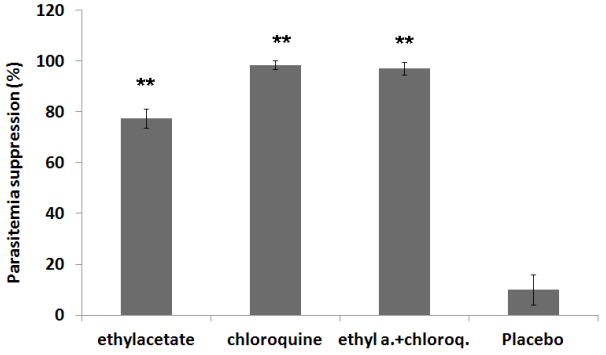

Figure 2. Parasitemia suppression (mean ± SEM) of infected mice treated with ethyl acetate fraction of saffron (700 mg/kg), chloroquine (20 mg/kg), ethyl acetate fraction (700 mg) + chloroquine (20 mg) and placebo on day 4 (1 day after cessation of treatment) in Peter's antimalarial test.

Table 1. Mean survival time (days) after injection (%) of infected mice treated with ethyl acetate fraction of saffron (700 mg/kg), chloroquine (20 mg/kg), ethyl acetate fraction (700 mg) + chloroquine (20 mg) and placebo

	Lived from 100%			
Group	Ethyl acetate fraction	Chloroquine	Ethyl acetate + chloroquine	Placebo
in day 7th	77.6	79.6	100.0	75.5
in day 14th	74.5	75.5	99.0	68.4
in day 28th	61.2	73.5	91.8	36.7

(Apocynaceae) exhibits antipyretic and antimalaria properties and has been used in the past for malarial treatment (16). The roots of *Cryptolepis sanguinolenta* from Asclepiadaceae family have been used in Africa as antimalarial drug. Cryptolepine and isocryptolepine are identified from these plants as responsible indoloquinoline alkaloids for this effect (17). The barks of Cinchona species a rich source of quinine and other quinolidine alkaloids were used for treatment of malaria fever from times long ago (18). The African antimalarial medicinal plants genus *Ancistrocladus* and *Triphyophyllum peltatum* contain naphthyl isoquiniline alkaloids like Yaoundamine and Habropetaline alkaloids with considerable in vitro antimalarial activity with good selectivity indices (19,20). Stem bark of *Pogonopus tubulosus* a tree from South America used traditionally in Bolivia against malaria and contains tubulosine indoline alkaloid with potent antimalarial activity in mice (21). *Glycyrrhiza glabra* a plant native to Iran contains licochalcone A and 18β-glycyrrhetinic acid with promised in vitro activities against *P. falciparum* (22,23). However, in comparison with previous reports on medicinal plants with antimalaria activity, it is the first report of *Crocus sativa* to be effective against *P. berghei*. This research was in agreement with another report recently published on 2 bioactive compounds isolated from *Crocus sativa* including crocin and safranal and their semi-synthetic derivatives with potent antimalarial activity in vitro against *P. falciparum* chloroquine sensitive strain with IC_{50} less than 20 μg/mL (9).

Ethyl acetate fraction of *Crocus sativus* stigma in combination with chloroquine exhibited a synergic pattern against chloroquine sensitive *P. berghei.* In comparison with other antimalarial plants, *Otostegia persica* combinational therapy with chloroquine revealed potentiating against the chloroquine sensitive and chloroquine resistant *P. berghei* strains (24). Artemisinin from *Artemisia anova* and mefloquine combination treatment has showed also marked synergism against *P. berghei* (25). But, *Artemisia aucheri*, an effective antimalarial plant, in combination with chloroquine has shown an antagonistic pattern (26).

The mechanism of action of saffron and its main bioactives crocin with carotenoid structure and safranal as an unsaturated monoterpenoid against malaria is not clear. In a cross-sectional study on malaria status between plasma carotenoids with structures very similar to crucin and indicators of disease severity like parasitemia, and antioxidant status with malaria, carotenoid concentrations were lower in the patients than in the control subjects (27). This study suggested that subjects with acute malaria have suppressed plasma concentrations of antioxidants, and that higher plasma carotenoids are associated with more and rapid clearance of malaria parasitemia (28,29). There are some reports also on immune modulation properties of *Crocus sativus* stigma (30). So, saffron as a rich source of crucin with carotenoid structure might act as a biological antioxidant supplement useful for reducing the parasitemia through alteration of oxidative stress and immune modulation especially in combination with malaria standard drugs. This study showed for the first time that saffron and its fractions has antimalarial activity as well as combination of its ethyl acetate fraction with chloroquine, which has a synergistic effect on chloroquine sensitive *P. berghei.* However, the exact mechanism of action of saffron, its fractions and its main bioactive like crocin and safranal in reducing blood parasite count is not clear and more studies are recommended in this regard.

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Authors' contributions

All contributed to the conception of the work, conducting the study, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. All the authors wrote the manuscript equally.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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