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Original article

Inhibition Test of Heme Detoxification (ITHD) as an Approach for Detecting Antimalarial Agents in Medicinal Plants

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Abstract

Background and objectives: There are several methods to assess the *in vitro* capability of heme inhibitory activity of antimalarial compounds; most of them require some specific equipment or toxic substances and sometimes the needed materials are not accessible. Regarding the necessity and importance of optimizing and standardizing experimental conditions, the present study has intended to improve the *in vitro* assessment conditions of the β-hematin formation inhibitory activity for screening herbal samples. **Methods:** Hemin, tween 20, and samples (9:9:2) were incubated in different conditions including: hemin concentration (30, 60, and 120 µg/mL), duration (4, 24, 48, and 72 h), pH of buffer (3.6, 4, 4.4, 4.8, and 5), and temperature (37 and 60 °C) in 96-well plates. Also, a total of 165 plant extracts and fractions were tested in the most suitable conditions. **Results**: The reaction time and the incubation temperature were determined as the critical factors. The effective conditions for β-hematin formation were found to be 60 °C after 24 h incubation. In this method, proper correlations with respect to negative (69%) and positive (67%) predictive values were obtained in comparison with the anti-plasmodial assay. Antimalarial activities of *Pistacia atlantica*, *Myrtus communis*, *Pterocarya fraxinifolia*, and *Satureja mutica* were found to correlate significantly with inhibition of the heme detoxification assay. **Conclusion:** These results support a rapid, simple and reliable approach for selecting and identifying a number of herbs for further related antimalaria investigations.

Keywords: fever; heme detoxification; Iranian traditional medicine; malaria

Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites. Approximately 3.3 billion people (half of the world's population) are at risk of malaria. According to the latest reports of WHO, there has been roughly 212 million malaria cases and an estimated 429000 malaria deaths in 2015 [1].

The malaria parasite digests hemoglobin in vacuole into amino acids and heme [2]. Free heme is toxic for the parasite [3] and *Plasmodium* has got several detoxification mechanisms such as hemozoin formation, heme-binding proteins

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and degradation of free heme by H_2O_2 to protect itself from toxic heme [4,5]. Some drugs show antimalarial effects via inhibition of hemozoin formation which is the most important mechanism for detoxification [6,7]. β-hematin, which is identical to hemozoin, is a synthetic polymer that is used for malaria in vitro assessments [8,9]. Identifying compounds that inhibit β-hematin formation is an approach for detecting antimalarial drugs. To date, various methods in measuring β-hematin formation inhibitory activity have been published; most of them require specific equipment [10,11] or toxic substances [12], and sometimes the needed materials are not available [7,13]. Huy et al. described a simple colorimetric inhibition assay of heme crystallization [14]. Regarding the necessity and importance of optimizing and standardizing experimental conditions, the present study has intended to improve the in vitro assay conditions of the β-hematin formation inhibitory activity for herbal samples. Also, medicinal plants, which were traditionally used to treat fever in Iranian traditional medicine, were screened by inhibition test of heme detoxification (ITHD).

Material and Methods

Plant material

The selected plants were the most commonly used in Iranian traditional medicine for treating diseases, especially fever [15]. Most of the specimens were collected from different places of Iran and identified at Traditional Medicine and Materia Medica Research Center (TMRC), Tehran, Iran; others were purchased from herbal market. Voucher specimens were deposited at the Herbarium of TMRC. The selected herbal samples have been mentioned in table 1.

Chemicals and reagents

Chloroquine diphosphate (Sigma, USA); DMSO (dimethyl sulfoxide), tween 20, sodium acetate, acetic acid (Merck, Germany); hemin (Alfa Aesar, Germany), were used in this study. The solvents for extraction and fractionation were provided from Mojalali Co. (Iran).

Extraction and fractionation

For preparing the crude extracts, 10 g of the dried powdered plants were macerated with constant shaking using 100 mL methanol for 24 h at room temperature. The fractions were prepared by macerating the dried powder or the residue of the plants in the solvent for 24 h at room temperature. Petroleum ether, chloroform and methanol were used successively for fractionation. The extracts and fractions were filtered and evaporated under vacuum. The dried extracts and fractions were stored at 4 °C.

Inhibition test of heme detoxification (ITHD)

The ITHD method was inspired from that previously described by Huy et al. [14]. In this method, some modifications were applied including: hemin concentration (30, 60, and 120 µg/ mL), duration (4, 24, 48, and 72 h), pH (3.6, 4, 4.4, 4.8, and 5), and temperature (37and 60 °C). The procedure for inhibition test was as follows; hemin was dissolved in dimethyl sulfoxide (DMSO). The solution was diluted freshly to 60 µg/ mL with 1 M acetate buffer (pH 4.8). Tween 20 was diluted to 0.012 g/ L with distilled water. Diluted hemin, tween 20, and samples which were solved by DMSO, were distributed in each well of a 96-well plate with ratio of 9:9:2, respectively, in triplicate. Final tested concentration of samples in each well was 200 µg/ mL. Also a control was prepared in triplicate under the same conditions, in the absence of hemin. The use of controls allowed to get rid of the residual interfering absorbance caused by the matrix of the sample. In negative control wells, the solvent (DMSO) used to solubilize the samples was added. Chloroquine diphosphate was used as the positive control. The plates were incubated at 60 °C for 24 h to allow completing the reaction. Finally, the absorbance was recorded with an ELISA reader at 405 nm. The results were calculated and expressed as percentage of heme detoxification inhibition.

Determination of performance of ITHD

This method was tested using a group of forty-five samples (table 1) most of them have been reported to show anti-plasmodial effects against *Plasmodium falciparum* [15-16]. The ITHD assay performance was evaluated by calculating the positive predictive value (PPV) and negative predictive value (NPV) according to the following equations:

PPV=number of true positives/(number of true positives + number of false positives)

NPV=number of true negatives/(number of true negatives +number of false negatives)

Results and Discussion

The aim of the current study was to develop a method to detect compounds with potential antimalarial activity. In the present study the ITHD method was modified from that previously described by Huy et al. [14]. The critical parameters identified for reaction were the reaction time (24 h) and the incubation temperature (60 °C). Chloroquine diphosphate is known to act as an inhibitor of heme polymerization. Under the conditions of ITHD, there was a direct relation between chloroquine concentration and percentage of inhibition of heme detoxification, and therefore the value of IC_{50} was obtained to be 14.1 ± 0.2 µM. Table 1 has presented the results of heme detoxification inhibition in forty-five herbal samples.

To evaluate the practical performance of the assay, we compared the results of inhibition test of heme detoxification with their anti-plasmodial activity (table 2) [15,16]. If percentage of heme detoxification inhibition was more than 90%, the assay was considered as positive whereas values less than 90% indicated a negative result.

Table1. The selected herbal samples investigated for inhibition test of heme detoxification (ITHD)

No.	Scientific name	Family	Plant part	Voucher number	Locality[a]	ITHD assay
1	*Astrodaucus orientalis* (L.) Drude.	Apiaceae	Fruit-bearing & Flowering branches	TMRC 1276	Tehran	-
2	*Berberis crataegina* DC.	Berberidaceae	Fruit-bearing branches	TMRC 1289	Khorassan	-
3	*Biebersteinia multifida* DC.	Geraniaceae	Tuber	TMRC 486	Khorassan-e-Shomali	-
4	*Bryonia aspera* Stev. ex Ledeb.	Cucurbitaceae	Tuber	TMRC 252	Golestan	+
5	*Buxus hyrcana* Pojark.	Buxaceae	Flowering branches	TMRC 1161	Gilan	-
6	*Caccinia macranthera* (Banks & Sol.) Brand	Boraginaceae	Root	TMRC 510	Golestan	-
7	*Capparis spinosa* L.	Capparaceae	Flowering branches	TMRC 1295	Golestan	-
8	*Centaurea bruguierana* (DC.) Bornm.	Asteraceae	Fruit-bearing branches	TMRC 1291	Khorassan-e-Shomali	-
9	*Centaurea golestanica* Akhani & Wagenitz	Asteraceae	Aerial Part	TMRC 730	Golestan	-
10	*Cephalanthera caucasica* Kranzl.	Orchidaceae	Flowering branches	TMRC 1288	Mazandaran	-
11	*Chenopodium botrys* L.	Chenopodiaceae	Fruit-bearing branches	TMRC 1331	Tehran	-
12	*Colutea persica* Boiss.	Fabaceae	Fruit-bearing branches	TMRC 1253	Mazandaran	-
13	*Dorema hyrcanum* Kos-Pol.	Apiaceae	Root	TMRC 414	Golestan	-
14	*Epilobium hirsutum* L.	Onagraceae	Aerial Part	TMRC 1694	Mazandaran	-
15	*Erodium oxyrhynchum* M. B.	Geraniaceae	Fruit-bearing & Flowering branches	TMRC 1277	Tehran	-
16	*Ferula oopoda* (Boiss. & Buhse) Boiss.	Apiaceae	Leaves	TMRC 970	Golestan	-

Table 1. Continued

No.	Scientific name	Family	Plant part	Voucher number	Locality[a]	ITHD assay
17	*Ferula oopoda* (Boiss. & Buhse) Boiss.	Apiaceae	Root	TMRC 970	Golestan	-
18	*Ferula szowitsiana* DC.	Apiaceae	Root	TMRC 965	Golestan	-
19	*Ficus carica* L.	Moraceae	Fruit-bearing branches	TMRC 1338	Mazandaran	-
20	*Glaucium oxylobum* Boiss. & Buhse	Papaveraceae	Flowering branches	TMRC 1283	Gilan	-
21	*Glycyrrhiza glabra* L.	Fabaceae	Young branch	TMRC 1170	Gilan	-
22	*Leontice leontopetalum* L.	podophyllaceae	Tuber	TMRC 1287	Khorassan-e-Shomali	-
23	*Linaria pyramidata* Lam.	Scrophulariaceae	Flowering branches	TMRC 1293	Khorassan	-
24	*Marrubium vulgare* L.	Lamiaceae	Flowering branches	TMRC 1286	Mazandaran	-
25	*Matricaria chamomilla* L.	Asteraceae	Aerial Part	HMS 330	Tehran	-
26	*Minuartia lineata* (Boiss.) Bornm.	Caryophyllaceae	Fruit-bearing branches	TMRC 1279	Mazandaran	-
27	*Myrtus communis* L.	Myrtaceae	Flowering branches	TMRC 1169	Gilan	+
28	*Ononis spinosa* L.	Fabaceae	Aerial Part	TMRC 1813	Azarbaijan-e-Sharghi	-
29	*Otostegia persica* (Burm.) Boiss.	Lamiaceae	Aerial Part	HMS 329	Sistan-va-Baluchestan	-
30	*Otostegia michauxii* Briq.	Lamiaceae	Aerial Part	TMRC 1152	Fars	-
31	*Paliurus spina-christi* Mill.	Rhamnaceae	Flowering branches	TMRC 1282	Mazandaran	-
32	*Papaver bracteatum* Lindl.	Papaveraceae	Flowering branches	TMRC 1297	Ardebil	-
33	*Parrotia persica* (DC.) C.A. Mey.	Hamamelidaceae	Bark	TMRC 1281	Golestan	-
34	*Perovskia abrotanoides* Karel.	Lamiaceae	Aerial Part	TMRC 801	Khorassan	-
35	*Phytolacca americana* L.	Phytolaccaceae	Aerial Part	TMRC 1154	Mazandaran	-
36	*Pistacia atlantica* (F. & C. A. Mey) Rech. F.	Anacardiaceae	Young branch	TMRC 1271	Gilan	+
37	*Pterocarya fraxinifolia* (Lam.) Spach	Juglandaceae	Fruit-bearing branches	TMRC 1167	Mazandaran	+
38	*Pteropyrum aucheri* Jaub. & Spach	Polygonaceae	Fruit-bearing & Flowering branches	TMRC 1285	Qazvin	+
39	*Rhamnus cornifolia* Boiss. and Hohen.	Rhamnaceae	Fruit-bearing branches	TMRC 1274	Kohgiluyeh-va-Boyer-Ahmad	-
40	*Roemeria refracta* DC.	Papaveraceae	Flowering branches	TMRC 1292	Khorassan	-
41	*Satureja mutica* Fisch. & C. A. Mey.	Lamiaceae	Aerial Part	TMRC 864	Golestan	+
42	*Scutellaria multicaulis* Boiss.	Lamiaceae	Flowering branches	TMRC 1273	Kohgiluyeh-va-Boyer-Ahmad	-
43	*Smilax excelsa* L.	Liliaceae	Aerial Part	TMRC 1511	Mazandaran	-
44	*Tamarix aralensis* Bunge	Tamaricaceae	Fruit-bearing & Flowering branches	TMRC 1284	Gilan	-
45	*Vitex pseudo-negundo* (Hausskn.) Hand.- Mzt.	Verbenaceae	Fruit-bearing & Flowering branches	TMRC 1153	Fars	-

[a] The province in Iran;

-: percentage of heme detoxification was less than 90%; +: percentage of heme detoxification was more than 90%

Extracts that inhibited *Plasmodium* growth by less than 50% at the concentration of 25 µg/ mL (IC_{50} > 25 µg/ mL) were grouped as being not significantly growth inhibitors. Although their antimalarial activity had been previously studied [15,16] to the best of our knowledge, their mode of action had not been published.

Table 2. Correlation between ITHD and the in vitro *Plasmodium falciparum* growth inhibition assay

		***In vitro P. falciparum* growth inhibition assay**	
		Positive (IC_{50} ≤25 µg/mL)	**Negative (IC_{50} >25 µg/mL)**
ITHD	Positive	4	2
	Negative	12	27

PPV value of 67% and NPV of 69% pointed out the good performance of the test in respect to the *in vitro P. falciparum* assay. Vargas *et al* reported good correlations in their method in respect with extracts PPV of 50% [17]. *Pistacia atlantica, Myrtus communis, Pterocarya fraxinifolia* and *Satureja mutica* showed significant inhibition through plasmodium growth and heme detoxification. False negative compounds may show their anti-plasmodial activity via other mode of actions. However, this method is a good predictor for screening antimalarial activity in compounds capable of interacting with heme. The advantage of ITHD assay is its simplicity and low cost for screening large samples, because it can be performed with basic laboratory equipment without handling *P. falsiparum*. A total number of 120 fractions from 40 plants were screened; 10% of fractions were heme inhibitors, and the methanol fractions were more effective compared to others. The results showed that the methanol fraction of *Glycyrrhiza glabra, Pistacia atlantica, Myrtus communis, Parrotia persica, Pteropyrum aucheri, Ferula oopoda, Roemeria refracta,* and *Tamarix aralensis*; the chloroform fraction of *Astrodaucus orientalis, Otostegia persica*; and the petroleum ether fraction of *Otostegia michauxii, Perovskia abrotanoides* could inhibit heme detoxification.

The present data demonstrated the action mode of three plants with anti-plasmodial activity including *G. glabra, P. atlantica* and *M. communis.*

The crude extract of *G. glabra* (licorice) did not show any inhibitory activity of heme detoxification at 200 µg/ mL, whereas it had shown anti-plasmodial activity against resistant strain K1 and antimalarial activity in vivo [15]; however, its methanol fraction showed considerable activity by ITHD. The use of a preliminary cleaning step removed a large part of the interfering compounds. This purification has, therefore, led to a significant improvement of the correlation between the hematin assay and the *P. falciparum* growth inhibition assay justifying the cost and labor related to this additional purification step [17]. Chen et al. reported licochalcone A isolated from Chinese licorice roots, inhibiting the in vitro growth of both chloroquine-susceptible (3D7) and chloroquine-resistant (Dd2) *P. falciparum* strains; they demonstrated that licochalcone A might be developed into a new antimalarial drug [18].

According to the results of the study, total extract and methanol fraction of *Pistacia atlantica* showed inhibitory effects on heme detoxification. Orhan *et al*. reported that the leaf extract was active against *P. falciparum* among thirteen lipophilic extracts prepared from various parts of *Pistacia vera* L. [19]. Adams et al. screened some plant extracts for antimalarial activity and found an ethyl acetate extract of *P. atlantica* was active. With subsequent isolation methods and structure elucidation, flavone 3-methoxycarpachromene was identified as the active substance [20]. So it may be considered as a selective plant for further antimalarial assays.

In our survey, flowering branches' extract of *M. communis* (myrtle) showed inhibitory effects. Also, methanol fraction of the species was effective. Milhau et al. showed that essential oils of leaves and stems of *M. communis* inhibited *P. falciparum* [21] and Naghibi et al. reported anti-plasmodial activity of *M. communis*, using the parasite lactate dehydrogenase assay; The IC_{50} values of *M. communis* extract were 35.44 and 0.87 µg/mL against K1 and 3D7 strains of *P.*

falciparum, respectively. Also the myrtle extract showed a significant inhibition in in vivo antimalarial assay [22]. These results support an approach for identifying plant-based agents with significant antimalarial effect.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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