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β-Hematin Formation Assay for Screening of Potential Antimalarial Activity of The Extracts and Fractions of three *Artemisia* Species

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In this study, *In-vitro* β-hematin formation assay, a spectrophotometric assessment is used to screen potential antimalarial effects of *Artemisia* extracts as well as their fractions. The n-hexane (n-hex), dichloromethane (DCM) and methanolic (MeOH) extracts obtained from aerial parts of *A. splendens* and *A. austriaca* by soxhlet apperatus, also DCM fractions, which were acquired by VLC method using n-hex, ethyl acetate (EtOAC) and MeOH, of *A. spicigera*, *A. splendens* and *A. austriaca* were examined by the mentioned method. N-hex and MeOH extracts of the herbs indicated weak anti-malarial activities, whereas, the DCM extracts of *A. spicigera* (IC_{50}= 0.99±0.01mg/ml, which had been reported in previous manuscript), *A. splendens* (IC_{50}= 1.93±0.09 mg/ml) and *A. austriaca* (IC_{50}= 1.15±0.04mg/ml) showed potent antimalarial effects. Among the all examined DCM fractions with different polarities, DCM F4'' (60% EtOAC/n-hex, the DCM fraction of *A. austriaca*) with IC_{50} value of 1.02 ± 0.03 mg/ml, DCM F5 (80% EtOAC/n-Hex, the DCM fraction of *A. spicigera*) with IC_{50} value of 1.21± 0.06 mg/ml, DCM F6 (100% EtOAC, the DCM fraction of *A. austriaca*) and DCM F7'' (100% MeOH, the DCM fraction of *A. austriaca*) with IC50 values of 1.44 ± 0.04 mg/ml for both, as well as DCM F7' (100% MeOH, the DCM fraction of *A. splendense*) with IC50 value of 1.58 ± 0.03 mg/ml indicated higher activities than the other DCM fractions. The mentioned fractions could be useful to bioassay-guided isolation of bioactive plant constituents.

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Introduction

Malaria infection, as a parasitic disease is the major cause of childhood mortality in developing countries. Approximately 83000 deaths were annually reported in all over the world [1]. Unfortunately, this vector- borne health problem is prevalent in south and southeastern areas of Iran [2, 3]. In different endemic areas, the main problem is the resistance of parasite species to antimalarial drugs, especially chloroquine [4]. Recognition of importance of the herbs has made growing interest in using these natural healthcare resources as a promising approach to control variety of disorders [5]. Artemisinins, as the most potent antimalarial phyto medicines, with formular structure of sesquiterpene lactone, were widely used to treat multidrug- resistant malaria, which was originally extracted from sweet wormwood (*Artemisia annua* L.) [6, 7]. So, this was resulted in drawing attention to the different species of *Artemisia* genus as antimalarial herbs. *Artemisia* genus belonging to Asteraceae (Compositeae) family which contains 34 species growing in Iran [8], comprises aromatic plants with anti- malarial, anti-hepatotoxic, anti- bacterial , antiviral , anti- fungal, insecticidal, analgesic, antiproliferative, anti- oxidant and cytotoxic activities [9-11]. Recently, screening of Iranian *Artemisia* species has been carried out for their potential antimalarial effects by *in-vitro* β-hematin formation assay [12-14]. *Artemisia spicigera* known as "*dermane ye sonbolei*" in Persian language, is an aromatic herb growing in Northern and Northwestern parts of Iran [15]. Previous researches revealed that among the extracts of *A. spicigera*, the DCM extract displayed the most potent antiplasmodial activity [16]. So, this study aimed to evaluate potential antimalarial activities of different fractions obtained from DCM extract of *A.spicigera*. Various extracts of *Artemisia splendens* known as "*dermane ye derakhshan*" in Persian language, have been shown different pharmacological effects. However, to the best of our knowledge, there are no reports regarding antimalarial effects of its extracts and fractions. Hence, either phytochemical or pharmacological studies have been carried out on extracts and essential oil of *Artemisia austeriaca*, known as "*dermane ye azari*" in Persian language, but according to our knowledge, no research has been conducted to evaluate antiplasmodial activity of this herb. Consequently, as part of our ongoing antimalarial study of the genus *Artemisia*, this paper will discuss potential antimalarial activities of *A.spicigera* DCM fractions, as well as both extracts and DCM fractions of *A. splendens* and *A. austeriaca*.

Material and Methods

Chemicals

The chemicals used to conduct this assay were as follows: Hematin procine, chloroquine diphosphate, sodium dodecyle sulfate (SDS), sodium acetate, magnesium sulfate, sodium hydrogen phosphate, sodium chloride (NaCl), potassium chloride (KCl), sodium hydroxide (NaOH), glucose and sodium bicarbonate purchased from Sigma-Aldrich Chemical Company. Also, oleic acid was purchased from Fluka, dimethyl sulfoxide and hydrochloric acid (HCl) from Merck and all the solvents used for extraction from Caledon and Scharlau.

Plant material

The aerial parts of *Artemisia spicigera* C. Koch, were collected from a place near the *Aras* river in *Jolfa* at E: 45° 17', N: 38° 39' (altitude of 700-750) at Eastern Azarbaijan province (Iran) during November 2009. The aerial parts of *Artemisia splendens* Willd. were gathered from *Kaleibar (gharedagh)* at E: 46° 48', N: 38° 49' (altitude of 2300) at Eastern Azarbaijan province (Iran) during June 2010. Also, the aerial parts of *Artemisia austeriaca* Jacq. were collected from *Yam* located in *Marand* at Eastern Azarbaijan province (Iran). The identity of the plants was confirmed by anatomical examination in comparison with the herbarium specimens (voucher nos. TBZ-FPH 716, TBZ-FPH 717 and TBZ-FPH 718, respectively) retained in the Pharmacy Faculty of Tabriz University of Medical Science, Tabriz , Iran.

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Extraction of the herbs and fractionation of DCM extracts

The air-dried and ground aerial parts of *A. spicigera*, *A. splendens* and *A.austeriaca* (120 g each) were individually Soxhlet extracted with n-hex, DCM and MeOH successively (1.1 L each). All of these extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45 °C. The yields of the extracts have been listed in **Table 1**.

Consequently, DCM extracts of the mentioned medicinal plants were fractionated by Vacuum Liquid Chromatography (VLC) with application of silica gel GF (20 g for each) and using the step gradient solvents with increasing polarities: n-hexane: ethyl acetate (90:10, 80:20, 60:40, 40:60, 20:80, 100:0) and finally 100% MeOH. At that time, all the obtained fractions were completely dried using a rotary evaporator at a maximum temperature of 45◦C. The yields of the DCM fractions have been listed in **Table 1**.

Heme bio crystallization and inhibition assay for evaluation of potential antimalarial activities

The potential antimalarial activity of the herbal extracts was evaluated by the method described by Afshar et al. with some modifications [14]. Briefly, varying concentrations of the extracts and DCM fractions (0-2 mg/mL in 10% DMSO) were incubated with 3 mM of hematin (freshly dissolved in 0.1 M NaOH), 10 mM oleic acid and 1 mM HCl. The reaction volume was adjusted to 1 mL using 500 mM sodium acetate buffer, pH 5. Chloroquine diphosphate was used as a positive control[16]. The samples were incubated overnight at 37 ◦C with regular shaking. After incubation, the samples were centrifuged (14,000 rpm, 10 min, at 21 °C) and the hemozoin pellets were repeatedly washed with incubation (15 min, at 37 °C; with gentle shaking) in 2.5% (w/v) SDS in phosphate buffered saline followed by a final wash in 0.1 M sodium bicarbonate, pH 9.0, until the supernatant was clear (usually 3-5 washes). After the final wash, the supernatant was removed and the pellets were re-suspended in 1 mL of 0.1 M NaOH before determining the hemozoin content by measuring the absorbance at 400 nm (Beckmann DU640 spectrophotometer) using a 1 cm quartz cuvette. The results were recorded as % inhibition (I %) of heme polymerization/crystallization compared to positive control (chloroquine) using the following equation: I% = [(AB–AA)/AB] × 100, where, AB: absorbance of blank; AA: absorbance of test samples. All experiments were conducted in triplicate measurements and presented as the Mean ± SD. Data were analyzed by Excel 2010 Microsoft. The IC_{50} and IC_{90} values were calculated from nonlinear regression analysis.

Results

Yields of aerial part extracts and the DCM fractions, as well as the results of the *in- vitro β*-hematin formation assay (IC_{50} and IC_{90} values), (n = 3), of the both aerial parts extracts and DCM fractions of *A. spicigera*, *A. splendens* and *A. austriaca* have been listed in **Table 1**. The extract yields of *A. spicigera* as well as the amounts of their antimalarial activities which were evaluated by *in-vitro β*-hematin formation assay have been reported in previous researches [18]. As shown in the **Table 1**, Two extracts (n-hex and MeOH) of the studied herbs did not indicate any significant anti-malarial activities while the DCM extracts of all the three herbs including *A. spicigera* (IC_{50}= 0.99±0.01, IC_{90}=1.63±0.02 mg/ml), *A. splendens* (IC_{50}= 1.93±0.09, IC_{90}=9.78±017 mg/ml) and *A. austriaca* (IC_{50}= 1.15±0.04, IC_{90}=1.90±0.04 mg/ml) compared to the positive control, chloroquine (IC_{50} = 0.04 ± 0.002, IC_{90} = 0.35 ± 0.006 mg/ml), showed potent antimalarial activities, that among which, DCM extract of *A. spisigera* showed the greatest activity (IC_{50}= 0.99 ± 0.01 mg/ml) . As presented in the **Table 1**, n-hex and MeOH extracts of *A. spicigera* as well as *A. splendens* indicated no antimalarial effects. Whereas, n-hex and MeOH extracts of *A. austriaca* possessed weak antimalarial activities with IC_{50} values of 3.19±0.36 and 2.15±0.10 mg/mL respectively, also, IC_{90} values of 2.15±0.10 and 3.25±0.09 mg/mL, successively. Among the seven DCM fractions with different polarities obtained from *A. spicigera,* DCM F5, DCM F6 and DCM F7 showed considerable anti-malarial activities with

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IC_{50} values of 1.21±0.06, 1.44± 0.04 and 1.78±0.01mg/mL as well as IC_{90} values of 1.45±0.01, 2.06 ± 0.03 and 1.92±0.01 mg/mL, successively. Among the seven different polarity fractions obtained from the DCM extract of *A. splendens* only DCM F7′ provided considerable anti-malarial activity with IC_{50} value of 1.58±0.03 and also IC_{90} value of 1.98±0.02 mg/ml. Moreover, among the DCM fractions obtained from the DCM extract of *A. austriaca*, DCM F3′′, DCM F4′′, DCM F5′′, DCM F6′′ and DCM F7′′ showed remarkable anti-malarial activities with IC_{50} values of 2.09±0.08, 1.02± 0.03, 1.66±0.07, 1.59±0.03 and 1.44±0.04 mg/mL as well as IC_{90} values of 3.27±0.18, 1.64 ± 0.03, 2.23±0.07, 2.13 ± 0.04 and 2.06±0.05 mg/mL, successively.

Table 1. The yields of the extracts and DCM fractions as well as 50% and 90% inhibition concentration values (mg/ml) of the tested *Artemisia* species by cell free *β*-hematin formation assay.

Herbs	Extracts/Fractions	Yields (%)	IC_{50} (Mg/Ml)[A]	IC_{90} (Mg/Ml)[A]
A. spicigera	n-hex[1]	6.41	-	-
	DCM[2]	1.45	0.99 ± 0.01	1.63 ± 0.02
	MeOH[3]	8.63	-	-
	10% EtOAC[4]/n-Hex (DCM F[5]1)	6.25	-	-
	20% EtOAC/n-Hex (DCM F2)	2.88	-	-
	40% EtOAC/n-Hex (DCM F3)	4.62	-	-
	60% EtOAC/n-Hex (DCM F4)	1.38	-	-
	80% EtOAC/n-Hex (DCM F5)	12.50	1.21± 0.06	1.45 ± 0.01
	100% EtOAC (DCM F6)	22.25	1.44 ± 0.04	2.06 ± 0.03
	100% MeOH (DCM F7)	33.88	1.78 ± 0.01	1.92 ± 0.01
A. splendens	n-hex	2.25	-	-
	DCM	0.47	1.93 ± 0.09	9.78 ± 0.17
	MeOH	12.00	-	-
	10% EtOAC/n-Hex (DCM F1′)	10.01	-	-
	20% EtOAC/n-Hex (DCM F2′)	4.50	-	-
	40% EtOAC/n-Hex (DCM F3′)	8.02	-	-
	60% EtOAC/n-Hex (DCM F4′)	5.50	-	-
	80% EtOAC/n-Hex (DCM F5′)	5.50	-	-
	100% EtOAC (DCM F6′)	8.02	-	-
	100% MeOH (DCM F7′)	41.50	1.58 ± 0.03	1.98 ± 0.02
A. austeriaca	n-hex	3.15	3.19 ± 0.36	4.08 ± 0.52
	DCM	1.25	1.15 ± 0.04	1.90 ± 0.04
	MeOH	11.34	2.15 ± 0.10	3.25 ± 0.09
	10% EtOAC/n-Hex (DCM F1′′)	3.75	-	-
	20% EtOAC/n-Hex (DCM F2′′)	4.85	-	-
	40% EtOAC/n-Hex (DCM F3′′)	13.50	2.09 ± 0.08	3.27 ± 0.18
	60% EtOAC/n-Hex (DCM F4′′)	19.90	1.02 ± 0.03	1.64 ± 0.03
	80% EtOAC/n-Hex (DCM F5′′)	15.65	1.66 ± 0.07	2.23 ± 0.07
	100% EtOAC (DCM F6′′)	9.85	1.59 ± 0.03	2.13 ± 0.04
	100% MeOH (DCM F7′′)	25.35	1.44 ± 0.04	2.06 ± 0.05
Chloroquine		-	0.04 ± 0.01	0.35 ± 0.01

[a] Experiment was performed in triplicate and the results were expressed as Mean ± SD.[1] n-hexane,[2] dichloromethane, [3] methanol, [4] ethyl acetate [5] fraction.

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Discussion

Hemoglobin is an important nutrient source for intra- erythrocytic malaria microorganisms. Due to its survival, hemoglobin catabolism occurs in an acidic digestive vacuole of infected erythrocytes that degrades host hemoglobin. As a result, hemoglobin proteolysis releases heme and generates amino acids. Also, free heme is released as a toxic byproduct of this process which could affect cellular metabolism. The heme moiety is stored as an inert polymer known as the malaria pigment of hemozoin, an insoluble, nontoxic and crystalline compound [18,19]. Therefore, the inhibition of hemozoin formation is an attractive target for development of several antimalarial drugs such as chloroquine and other quinoline antimalarials considered as a suitable target for drug screening programs [20]. Herbal derived compounds offer an approach to discovery of analogues and parent antiplasmodial agents. Such trials include evaluation of the potential antimalarial activities of the herbal extracts, fractions and isolated phyto-constituents purified from the extracts. At this point, the requirement for low-cost and simple ways of drug discovery seems to be necessary [21, 22]. So, *In-vitro* assays based on spectral characteristics and differential solubility of monomeric heme and *β*-hematin (synthetic analogue of hemozoin) have been described and used for screening of novel synthetic and natural antimalarial agents [23, 24].

A survey of Traditional Persian Medicine (TPM) manuscripts showed that "Afsantin" (*Artemisia absinthium* L.), "Qaysum" (*Artemisia abrotanum* L.) and "Shih" (*Artemisia santonicum* L.) had been used to treat "tabe rebá" (malaria like fever) [25]. So the connection between TPM and the results of the current studies about anti-palasmodial activities of *Artemisia* species [13, 14, 17] further leads us to evaluate antimalarial effects of the various species of the mentioned genus growing in Iran.

According to the previous researches, the isolated bioactive compounds from DCM extracts of diverse *Artemisia* species, such as terpens, essential oils, lignans, coumarins, sesquiterpene lactones have indicated anti-plasmodial activities [26-30]. Since, artemisinin antimalarials do not inhibit hemozoin formation [31] and artemisinin along with flavonoids have involved in the antimalarial activity of *A. annua*, thus, the mechanism of action might be ascribed to phyto-complex not a selected group of bioactive phytochemicals. As mentioned before, the DCM extracts of the mentioned herbs were selected for further investigations due to their potent antimalarial activities and then they were subjected to fractionation by the above-mentioned procedures. As it could be seen in the **Table 1**, among the seven different polarity fractions of *A. spicigera* (DCM F5, F6 and F7), *A. splendens* (DCM F´7) as well as *A. austriaca* (DCM F´´3, F´´4, F´´5, F´´6 and F´´7) rather polar fractions illustrated anti plasmodial activities, which might be attributed to the presence of methoxylated flavonoids as well as methylated coumarins [12]. Among the DCM extracts and the related fractions of the tested herbs, DCM extract of *A. spicigera* showed considerable antimalarial activity with IC_{50} and IC_{90} values of 0.99 ± 0.01and 1.63 ± 0.02 mg/ml respectively. Furthermore, in contrast to *A. splendens* and *A.austriaca*, anti-plasmodial activity of DCM extract of *A.spicigera* was more potent in comparison to its DCM fractions, which could be ascribed to synergistic effect of various groups of active ingredients, which have been extracted in fractions with different polarities.

Moreover, among the all examined DCM fractions, DCM F4'' with IC_{50} value of 1.02 ± 0.03 mg/ml, DCM F5 with IC_{50} value of 1.21± 0.06 mg/ml and DCM F6 and DCM F7'' with IC_{50} values of 1.44 ± 0.04 mg/ml for both indicated more potent anti-plasmodial activities, which could be of practical use for bioassay-guided isolation of bioactive phytochemicals.

Conclusion

It be concluded that among the extracts of the examined *Artemisia* species, the DCM extracts displayed the considerable antimalarial activities. Besides, among DCM fractions of which, rather polar fractions illustrated anti- plasmodial effects examined by *in-vitro β*-hematin cell free method. Overall, the results of this preliminary study persuaded us not only to focus on purifying the active components of more potent DCM fractions

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but also to investigate on animal models for *in-vivo* evaluation.

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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