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Original article

Evaluation of anti-malarial activity of *Artemisia turcomanica* and *A. kopetdaghensis* by cell-free β-hematin formation assay

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Abstract

Background and objectives: The plants of genus *Artemisia* (Asteraceae) have been conventionally used for prevention and medication of a number of ailments. In the present research, ten extracts with different polarities from aerial parts of two *Artemisia* species, *A. kopetdaghensis* and *A. turcomanica* were evaluated for their potential anti-malarial properties. **Methods**: The plant materials were extracted successively with petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAC), ethanol, and ethanol-water (1:1 v/v) by cold maceration method. Cell free *β*-hematin formation assay were used for assessing anti-malarial activity of obtained extracts. **Results***:* DCM extract of *A. kopetdaghensis* and PE extract of *A. turcomanica* showed remarkable anti-malarial activity with IC_{50} values of 1.04±0.02 mg/mL and 0.90±0.27 mg/mL, respectively, compared to positive control (chloroquine, IC_{50} 0.04±0.01 mg/mL). **Conclusion:** It seems that the anti-malarial activity of these extracts might be bound up with the presence of compounds with low or medium polarity; hence, this preliminary test indicated that these potent extracts could be considered for further investigations to find new sources of anti-malarial phytochemicals.

Keywords: anti-malaria, *Artemisia kopetdaghensis*, *Artemisia turcomanica*, cell-free β-hemation formation assay

Introduction

Members of genus *Artemisia* (from the tribe Anthemideae, family Asteraceae) have been noteworthy medicinal plants throughout the world especially since the discovery of artemisinin isolated from *A. annua* as well as its successful clinical effects in treatment of malaria [1,2]. Artemisinin or qinghaosu with its endoperoxide sesquiterpene lactone structure has been a well-known potent anti-malarial drug, even against chloroquine- and quinine- resistant *Plasmodium falciparum* during the last decades [3,4]. Artemisinin is capable of killing the parasite in all stages by interaction with toxic free heme which damages parasite membranes [5]. Moreover, this natural compound can block free heme biocrystalization (like chloroquine and

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other 4-aminoquinolines) and hemoglobin degradation [6]. Nowadays, by emerging of artemisinin-resistant parasites in some places [7-9], scientists have started to conduct new investigations on medicinal plants in order to find novel natural anti-malarial drugs [10-13]. In regard to these researches, notable range of natural compounds such as sesquiterpenes [14,15], diterpenes [16,17], steroids [18], flavonoids [19,20], alkaloids [21-24], stilbenes [25-27] and coumarin derivatives [28,29] have displayed anti-malarial properties in various *in vitro* tests. In our recent studies on *Artemisia* genus, dichloromethane extracts of *A. spicigera*, *A. scoparia*, *A. aucheri*, *A. armeniaca* and *A. ciniformis* along with ethyl acetate extracts of *A. turanica* and *A. biennis* showed remarkable anti-malarial activity in cell free β-hematin formation assay [30-32]. In continuation of our researches on Iranian *Artemisia* species, different extracts of *A. turcomanica* and *A. kopetdaghensis* were screened for their anti-malarial effects. *A. turcomanica* is a perennial herb which has been used widely in Iranian and Chinese folk medicine [33]. Recently, the essential oil of *A. turcomanica* was reported to have anti-proliferative and anti-bacterial activities [33-35].

Artemisia kopetdaghensis, with its local name *'ushan'*, is an aromatic plant which has been used in Iranian herbal medicine for its antimicrobial, antifungal, anti-inflammatory and sedative properties [36,37]. Furthermore, in other studies, cytotoxic effects of the methanol and hydro-ethanol extracts of *A. kopetdaghensis* as well as its essential oil have been demonstrated [38,39]. To the best of our knowledge, there has been no report about anti-malarial activity of these plant extracts; hence, based on our previous positive results about *Artemisia* species, we conducted the current research for evaluating the anti-malarial activity of the mentioned plants.

Experimental

Chemicals

Hematin porcine, chloroquine diphosphate, sodium chloride, magnesium sulfate, sodium dodecyl sulfate (SDS), sodium acetate, , sodium hydrogen phosphate, potassium chloride, sodium hydroxide, glucose, and sodium bicarbonate were purchased from Sigma-Aldrich Chemical Company (Germany), oleic acid from Fluka (India), dimethylsulfoxide (DMSO), HCl from Merck (Germany), and all the solvents utilized for extraction from Caledon (Canada) and Scharlau (Spain).

Plant material

The aerial parts of *A. turcomanica* Gand. were collected near Shahr abad (953 m height), Bojnord, North Khorasan province, north east of Iran during October 2010. The identity of the plant was confirmed by Dr. Valiollah Mozaffarian, from Research Institute of Forest and Rangelands, Tehran, Iran. A voucher specimen (No. 12573) was retained at the Herbarium of School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. Aerial parts of *A. kopetdaghensis* Krasch., M. Pop. & Lincz. ex Poljak. were collected from Baba aman rural district (1034 m height), Bojnord, North Khorasan province during October 2010 and authenticated by Dr. Valiollah Mozaffarian. A voucher specimen (No. 12571) was deposited at the above mentioned Herbarium.

Extraction

The plant materials were air-dried at room temperature under shade, finely ground, and extracted by cold maceration method. Each plant sample (100 g) was extracted successively with petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAC), ethanol, and ethanol-water (1:1 v/v) at room temperature (sequential maceration with ca. 3 × 1 L of each solvent). All extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45 °C.

Cell-free β-hematin formation assay

The anti-malarial activity of extracts was assessed by the *in vitro* cell-free *β*-hematin formation assay explained by Afshar *et al.* [30]

with some necessary modifications. Concisely, 10 µL different concentrations (0.4-2.0 mg/mL in DMSO) of each extract were mixed with 100 µL of hematin (3 mM), 10 µL oleic acid (10mM) and 10 µL HCl (1M). The final amount was adjusted to 1 mL using sodium acetate buffer, pH 5. Chloroquine diphosphate was considered as a positive control. The reaction mixtures were incubated overnight at 37 °C with constant gentle shaking. Incubation was terminated by centrifugation (14000 rpm, 10 min, at 21 °C) to collect the *β*-hematin pellets. The pellets were repeatedly washed with incubation (15 min at 37 °C with constant shaking) in 2.5% (w/v) SDS in phosphate buffer saline followed by a final wash in 0.1 M sodium bicarbonate, until the supernatant was colorless. To determine the heme amount crystallized into *β*-hematin, the pellets were dissolved in 0.1 M NaOH before recording the absorbance at 400 nm DU640 spectrophotometer, USA). The results were calculated as % inhibition (I%) of heme crystallization compared to negative control (DMSO) using the following equation: I% = [(AN–AS)/AN]×100; AN: absorbance of negative control; AS: absorbance of test samples.

Statistical analysis

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 and Discussion

Table.1 shows a summary of anti-malarial data from cell-free *β*-hematin formation assay which was carried out on ten different extracts from aerial parts of *A. turcomanica* and *A. kopetdaghensis*. The inhibition of *β*-hematin formation illustrated as percentage (*I* %) and standard deviations (*n*=3) are calculated for each plant extract. The amount of IC_{50} and IC_{90} values were determined graphically by plotting concentrations versus percentage of inhibition. The solvent of extracts (DMSO) was utilized as the negative control and chloroquine was applied as the positive control.

As shown in table.1, in comparison to the standard anti-malarial compound, chloroquine (IC_{50} = 0.04 ± 0.01 mg/mL, IC_{90} = 0.35 ± 0.01 mg/mL), PE extract of *A. turcomanica* possessed the most potent anti-malarial activity with IC_{50} and IC_{90} values of 0.90 ± 0.27 mg/ml and 1.62 ± 0.68 mg/ml, respectively. In the case of *A. kopetdaghensis*, DCM extract of this plant was found to be more potent (IC_{50} 1.04±0.02 mg/mL, IC_{90} 1.20±0.02 mg/mL) than other extracts. Moreover, good potencies were displayed by DCM extract of *A. turcomanica* (IC_{50} 1.95±0.07 mg/mL, IC_{90} 2.69±0.11 mg/mL), and ethanol extracts of both plants with IC_{50} and IC_{90} values of 1.99±0.05 mg/mL and 2.26 ± 0.06 mg/mL for *A. turcomanica* and, 2.64±0.21 mg/mL and 4.09±0.37 mg/mL for *A. kopetdaghensis*, respectively. Hydro-alcohol and EtOAC extracts of both plants showed no anti-malarial activity.

Table.1. The inhibitory effect of *Artemisia turcomanica and A. kopetdaghensis* extracts in cell-free *β*-hematin formation assay

Plants	Extracts/Fractions	Yields (%)	IC_{50} (mg/mL)[a]	IC_{90} (mg/mL)[a]
Artemisia turcomanica	Petroleum ether (PE)	3.75	0.90 ± 0.27	1.62 ± 0.68
	Dichloromethane (DCM)	9.18	1.95 ± 0.07	2.69 ± 0.11
	Ethyl acetate (EtOAC)	0.90	-	-
	Ethanol	2.57	1.99 ± 0.05	2.26 ± 0.06
	Hydro-alcohol	15.87	-	-
Artemisia kopetdaghensis	Petroleum ether (PE)	4.53	-	-
	Dichloromethane (DCM)	10.24	1.04 ± 0.02	1.20 ± 0.02
	Ethyl acetate (EtOAC)	0.76	-	-
	Ethanol	3.46	2.64 ± 0.21	4.09 ± 0.37
	Hydro-alcohol	19.24	-	-
Chloroquine	-	-	0.04 ± 0.01	0.35 ± 0.01

a: IC_{50} and IC_{90} represent the concentration of the tested samples that was required for 50% and 90% inhibition, respectively.

Malaria represents the most life-threatening parasitic disease which is caused by the reproduction of the parasite *Plasmodium falciparum* in host erythrocyte [40]. During blood stage, the parasite utilizes hemoglobin as its primary nutrition source for its development and proliferation [41,42]. Subsequently, heme is released which is harmful for the parasite; hence, the *plasmodium* detoxifies free heme through different pathways, dominantly via biocrystalization of heme into hemozin or malaria pigment [43,44]. Thus, prevention of heme biocrystalization has been considered as a unique drug target in anti-malarial drug discovery as well as screening programs [45,46]. In the present study, differential solubility of monomeric heme and β-hematin (synthetic homologue of hemozoin) as well as their spectral characteristics was established to form the basis of cell free β-hematin formation assay [30-32]. Among ten tested extracts, PE extract of *A. turcomanica* showed the most potent activity in inhibition of heme biocrystalization which might be due to the presence of low-polar pharmacologically active constituents like sesquiterpenes, diterpenes and steroids. Since the aerial parts extract of *A. turcomanica* contain germacranolide sesquiterpene lactones such as artemin, artemorin, gallicin, anhydrovelotorin, ridentin acetate and 11β, 13dihydro-redentin acetate [47], it is very likely that these phytochemicals might have contributed chiefly to the observed anti-malarial effect of PE extract of *A. turcomanica*. Moreover, the present results showed that DCM extract of *A. kopetdaghensis* had a high anti-malarial activity. To the best of our knowledge, there is no report on phytochemical analysis of the extracts of this plant; however, it seems that its potent anti-malarial effect might be due to the presence of terpenoid components which have been indicated to possess anti-malarial properties [14-17]. In addition, the anti-malarial activity demonstrated by the ethanolic extracts of both *Artemisia* species may be due to the presences of saponins and tannins [48] as well as eliminating as much the lipid compounds causing synergistic effect with oleic acid in assay [30] As depicted in figure 1 and in line with our previous reports, the values of inhibition percentage were negative at lower concentrations of the potent extracts. Perhaps there are lipid compounds in the potent extracts that cause the synergistic effect with oleic acid in the assay; therefore, the observed absorbance were higher than the negative control [30-32]; however, by thoroughly removing the lipids and fatty acids, the values of the IC_{50} and IC_{90} remarkably decreased.

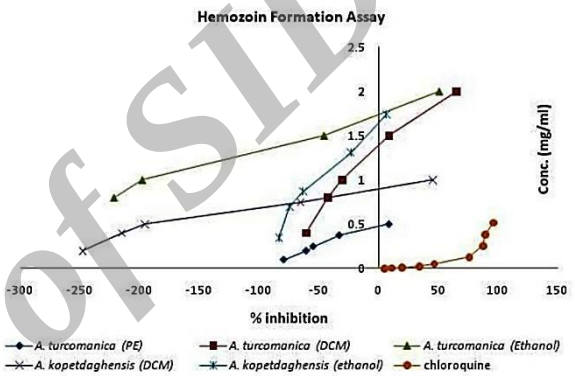

Figure 1: Comparison of %inhibition of heme biocrystallization between active extracts of *A. turcomanica*, *A. kopetdaghensis* and chloroquine solution in cell-free *β*-hematin formation assay

The present research showed that the PE extract of *A. turcomanica* possessed greater potential to be explored for production of novel antimalarial phytomedicines. This preliminary research and its significant data convinced us to concentrate for further fractionation of the extract and then isolation and structure elucidation of the active components as well as *in vivo* anti-malarial evaluations.

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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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