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In vitro antimalarial activity of different extracts of *Eremostachys macrophylla* Montbr. & Auch.

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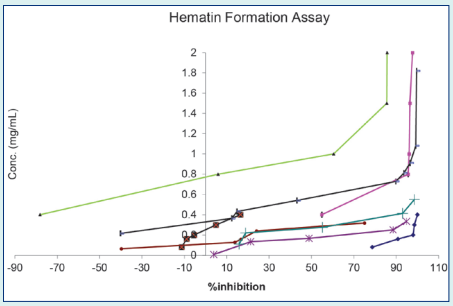
Abstract

Introduction: The risk of drug resistance and the use of medicinal plants in malaria prevention and treatment have led to the search for new antimalarial compounds with natural origin.

Methods: In the current study, six extracts with different polarity from aerial parts and rhizomes of *Eremostachys macrophylla* Montbr. & Auch., were screened for their antimalarial properties by cell-free β-hematin formation assay.



Results: Dichloromethane (DCM) extracts of both parts of plant showed significant antimalarial activities with IC_{50} values of 0.797 ± 0.016 mg/mL in aerial parts and 0.324 ± 0.039 mg/mL in rhizomes compared to positive control (Chloroquine ,IC_{50} = 0.014 ± 0.003 mg/mL, IC_{90} = 0.163 ± 0.004 mg/mL). Bioactivity-guided fractionation of the most potent part (DCM extract of rhizomes) by vacuum liquid chromatography (VLC) afforded seven fractions. Sixty percent ethyl acetate/n-hexane fraction showed considerable antimalarial activity with IC_{50} value of 0.047 ± 0.0003 mg/mL.

Conclusion: From 6 extracts with different polarity of *E. macrophylla*'s aerial parts and rhizomes, the DCM extract of both parts were the most active extract in this assay. The preliminary phytochemical study on the VLC fractions of the most potent part persuades us to focus on purifying the active components of these extracts and to conduct further investigation towards in vivo evaluation.

Introduction

Malaria as one of the oldest recorded diseases is considered as the major parasitic disease in tropical and subtropical areas of the world, imposing significant morbidity and often mortality where they occur.[1,2] Each year more than 216 million new cases and approximately 655000 deaths of malaria are diagnosed and recorded, most of which are children.[3,4] This global ailment is caused by *Plasmodium* species, of which *P. falciparum* is the most deadly pathogen, and is accountable for over 85% of cases.[5,6] In Iran, malaria is one of the most crucial parasitic diseases in the south-eastern areas, responsible for around 95% of all malaria cases in the country.[3]

Chloroquine was synthesized for the first time in 1934 and designated as the chosen drug for treatment of malaria in 1946. It is known as the cheapest and commonly used drug for the treatment of malaria.[5,7,8] Chloroquine is thought to display its antimalarial activity by inhibiting the crystallization of toxic heme produced during proteolysis of hemoglobin in the parasite vacuoles.[9] Unfortunately, this medication has become ineffective due to the generation of chloroquine-resistant species of the malaria parasite.[5,7,8] Nowadays, medicinal plants, as a new resource, are popular and utilized in the prevention and treatment of malaria in different parts of the world.[6-8,10,11] For instance, artemisinin as a natural product from an indigenous herb in China, was derived from the plant *Artemisia annua* in 1972. It has been growing attention to this plant since the isolation of artemisinin because of its distinguished clinical effects as a potent antimalarial agent.[7,12-14] Artemisinin with an endoperoxide sesquiterpene lactone structure can abolish parasite of all stages by a reductive interaction with free heme, resulting in generation of some types of free radicals that could alkylate parasite proteins and

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damage membranes. Artemisinin also acts by blocking free heme biocrystallization (like 4-aminoquinolines) and hemoglobin degradation.[14] World Health Organization (WHO) recommended artemisinin-based combination therapy (ACT) as the first-line treatment of unsophisticated malaria in April 2002.[15] In the last few years, WHO has recommended withdrawal of the oral artemisinin-based monotherapies from the market due to the risk of emergence of artemisinin-resistant parasites;[14,16] therefore, several new researches have been conducted to find new natural sources of antimalarial drugs.[17-21] Based on these investigations, remarkable diversity of new natural products such as coumarin derivatives,[22,23] flavonoides,[24,25] stilbenes,[26-28] sesquiterpenes,[29,30] diterpenes,[31,32] steroids,[33] alkaloids,[34-37] etc. showed antiplasmodial activities in different in vitro assays.

In the current study, for the first time, different extracts of *E. macrophylla* were evaluated from antimalarial effects viewpoints. *E. macrophylla* (family: Lamiaceae alt. Labiatae; subfamily: Lamioideae) is one of the 15 endemic Iranian species of the *Eremostachys* genus. It is a perennial plant with bulky rhizomes.[38] According to previous studies containing traditional knowledge and clinical findings, a number of *Eremostachys* species are applied as local analgesic and anti-inflammatory agents. Also this genus has shown antinociceptive, antidepressant and antibacterial activities.[39-44] The main objectives of this study were to investigate on (a) the antimalarial activity of different extracts of aerial parts and rhizomes of this plant, (b) fractionation of the most potent extract, (c) determination of the most potent fractions, and (d) identification of the chemical composition of them by GC-MS.

Materials and methods

Chemicals

All the solvents used for extraction and fractionation were purchased from Caledon Labs (Halton Hills, Ontario, Canada). Hematin porcine, chloroquine diphosphate, sodium dodecyl sulfate (SDS), sodium acetate, magnesium sulfate, sodium hydrogen phosphate, sodium chloride, potassium chloride, sodium hydroxide, glucose, and sodium bicarbonate were purchased from Sigma-Aldrich Company Ltd (Dorset, United kingdom). Oleic acid was obtained from Fluka (Gujarat, India) and dimethylsulfoxide, hydrochloric acid, and silica gel 60 (0.040–0.063 mm) from Merck company (Darmstadt, Germany).

Plant material

The aerial parts and rhizomes of *E. macrophylla* Montbr. & Auch. were collected respectively during July and September–October 2012 from Sahand mountains in East Azarbaijan province in Iran [37.759 (37° 45' 32.4" N) latitude 45.9783 (45° 58' 41.9" E) longitude and altitude 1950 m above sea level]. A voucher specimen (Tbz-FPh.739) has been deposited in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Extraction

Air-dried and ground aerial parts and rhizomes of *E. macrophylla* (100 g each) were Soxhlet extracted respectively with n-Hexane, DCM, and methanol (MeOH) (1 L each). All obtained extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45°C.

Fractionation

An amount of 1.1 g of DCM extract from rhizomes of *E. macrophylla* was fractionated by vacuum liquid chromatography (VLC) over silica gel (20 g) with solvent mixtures of increasing polarities: ethyl acetate/n-hexane (i.e., at ratios of 10:90; 20:80; 40:60; 60:40; 80:20; 100:0) and methanol. All the fractions were fully dried using a rotary evaporator at a maximum temperature of 45°C.

Antimalarial cell free assay

Antimalarial activity of plant extracts was evaluated by the method explained by Afshar et al[45] with some modifications. Briefly, varying concentrations (0–2 mg/mL in DMSO) of the extracts and fractions were produced. The reaction mixtures were incubated with 3 mM of hematin, 10.0 mM oleic acid, and 1 M HCl. The final volume was adjusted to 1.0 mL volume using sodium acetate buffer, pH 5, overnight at 37°C with constant gentle shaking. Chloroquine diphosphate was used as a positive control. After incubation, samples were centrifuged (14000 rpm, 10 min, at 21°C) and the hemozoin pellet was repeatedly washed with incubation (15 min at 37°C with regular shaking) in 2.5% (w/v) SDS in phosphate buffered saline followed by a final wash in 0.1 M sodium bicarbonate until the supernatant was clear (usually 3–8 washes). After the final wash, the supernatant was discarded and the pellets were dissolved in 1.0 mL of 0.1 M NaOH before determining the hemozoin content by measuring the absorbance at 400 nm (Spectronic Genesys spectrophotometer). The results were recorded as % inhibition (*I*%) of heme crystallization compared to negative control (DMSO) using the following equation:

$I\% = [(AN-AS)/AN]\times100$, where, AN is absorbance of negative control and AS is absorbance of test samples.

GC-MS analysis of potent fractions

GC–MS analyses were carried out on a Shimadzu QP-5050A GC–MS system (Shimadzu Corp., Kyoto, Japan) equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 µm); oven temperature, rising from 100°C to 310°C at a rate of 5°C; injector temperature, 280°C; carrier gas, helium at a flow rate of 1.0 mL/min; split ratio, 1:19; ionization energy, 70 eV; scan time, 1 s; mass range, 30–600 amu.

Identification of components

Identification of the components was based on direct comparison of the retention times and mass spectral

data with those for standard compounds, and computer matching with the NIST 21, NIST 107, and WILEY229 library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature (Adams 2004).

Statistical analysis

All experiments were performed in triplicate and presented as the Mean ± SD. Data were analyzed by Microsoft Excel 2010. The IC_{50} and IC_{90} values were calculated by nonlinear regression analysis.

Results

The results from the cell free β-hematin formation assay of six different extracts from aerial parts and rhizomes of *E. macrophylla* and seven fractions of the most potent extract as well as the extraction and fractionation yields are listed in Table 1. The inhibition of β-hematin formation represented as percentage (*I* %) and standard deviations ($n = 3$) are considered for each extract/fraction. The values of IC_{50} and IC_{90} were measured graphically by plotting concentrations versus percentage of inhibition. Chloroquine was used as positive control with the most potent antimalarial activity and the extracts solvent (DMSO) was used as negative control without any antimalarial activity.

As shown in Table 1, n-Hexane and methanol extracts of aerial parts and rhizomes had no antimalarial activity at all while the DCM extracts of both parts especially DCM extract of rhizomes showed the most potent antimalarial activity compared to the standard antimalarial compound, chloroquine (IC_{50} = 0.014 ± 0.003 mg/mL, IC_{90} = 0.163 ± 0.004 mg/mL). IC_{50} values of DCM extract of aerial parts and rhizomes were 0.797 ± 0.016 and 0.324 ± 0.039 mg/mL, respectively. This was 0.863 ± 0.018 and 0.753 ± 0.009 mg/mL for IC_{90} values of this extract, respectively.

As shown in Table 1 and Fig. 1, after fractionation of the most potent extract (DCM extract of rhizomes) by VLC method, the results showed that among the seven different polarity fractions, 60% ethyl acetate/n-hexane fraction had considerable antimalarial activity with IC_{50} value of 0.047 ± 0.0003 and IC_{90} value of 0.249 ± 0.023 mg/mL.

Polar fractions of VLC (80% ethyl acetate/n-hexane, 100% ethyl acetate and MeOH fractions) demonstrated remarkable antimalarial effects with close IC_{50} and IC_{90} values (Table 1).

Table 1. The 50% and 90% inhibition concentrations (mg/mL) of active extracts and VLC fractions of DCM extract of *E. macrophylla* rhizomes in β-hematin formation assay

Extracts/fractions	Yields (%)	IC_{50} (mg/mL)	IC_{90} (mg/mL)
DCM (Rhizomes)	0.32	0.324 ± 0.039	0.753 ± 0.009
DCM (Aerial parts)	0.43	0.797 ± 0.017	0.863 ± 0.018
Fr.10%	0.591	1.319 ± 0.196	4.163 ± 0.585
Fr. 20%	Trace	*	*
Fr.40%	0.55	*	*
Fr.60%	1.91	0.047 ± 0.0003	0.249 ± 0.023
Fr.80%	7.67	0.228 ± 0.002	0.379 ±0.021
Fr.100%	9.42	0.217 ± 0.001	0.432 ± 0.022
Fr.MeOH	68.18	0.576 ± 0.011	0.857 ± 0.052
Chloroquine	Positive control	0.014 ± 0.003	0.163 ± 0.004

Experiment was performed in triplicate and expressed as Mean ± SD.
* No effect.

Discussion

Malaria is a life-threatening disease caused by the *Anopheles* mosquitoes that infects the human body by intracellular parasite of the genus *Plasmodium*.[46] *Plasmodium falciparum* utilizes host hemoglobin as a main nutrient source for its growth and reproduction. This parasite ingests more than 75% of the host hemoglobin during intra-erythrocytic cycle; therefore, substantial amounts of heme is generated as a toxic by-product from massive degradation of hemoglobin.[47,48] Subsequently, parasite for protecting itself from the self-produced toxic material has evolved a process through the crystallization of heme into an insoluble, nontoxic crystalline pigment known as hemozoin.[14,49,50] Hemozoin is equivalent to β-hematin, which consists of cyclic heme dimers arranged in an ordered crystalline structure through intermolecular hydrogen bonding. It is believed to be the most validated target of detoxification. Therefore, the inhibition of hemozoin formation is a way for drug screening programs. Many different quantitative in vitro methods based on spectral characteristics and differential solubility of monomeric heme and β-hematin has been used for evaluation of antimalarial activities.[48,51-53] In this study, 6 extracts and 7 fractions of aerial parts and rhizomes of *E. macrophylla* were evaluated for their antimalarial activity by an in vitro method. Our findings revealed that the n-hexane and the MeOH extracts of both parts of *E. macrophylla* did not show any significant antimalarial activities but the DCM extracts of both aerial parts and rhizomes showed significant potential antimalarial effects in comparison with negative control. It was indicated that potent antimalarial constituents of active extracts might possess medium polarity. Among two DCM extracts of *E. macrophylla*, DCM extract of rhizomes demonstrated the most potent activity (IC_{50} =0.324 ± 0.039 mg/mL , IC_{90} = 0.753 ± 0.009mg/mL) which guided to fractionate it by VLC over silica gel with solvent mixtures of increasing polarities (Fig. 2). Based upon the results presented in Table 1 and Fig. 1, among the seven fractions obtained by VLC, the 60% ethyl acetate/n-hexane fraction was the most potent fraction with IC_{50} value of 0.047 ± 0.0003 and IC_{90} value of 0.249 ± 0.023 mg/mL. Other polar fractions, 80% ethyl acetate/n-hexane, 100% ethyl acetate/n-hexane and 100% MeOH, also were determined as significantly potent antimalarial fractions.

GC-MS analysis of fractions with antimalarial activity indicated the presence of steroids, polycyclic aromatic hydrocarbon, diterpenoid derivatives, linear alcohols and fatty acids as major constituents. Previous investigations showed that terpenes, steroids, coumarins, flavonoids,

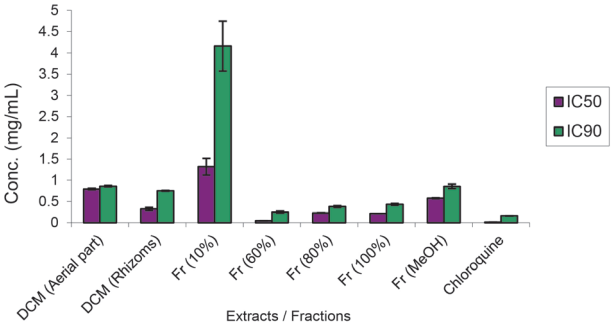

Fig. 1. Comparison of IC_{50} and IC_{90} values (mg/mL) of active extracts of *E. macrophylla,* fractions of the most potent extract and chloroquine solution in β-hematin formation assay. The values were reported as Mean± SD.

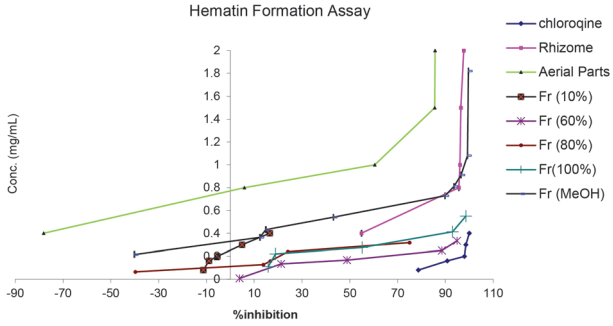

Fig. 2. Comparison of % inhibition of heme crystallization between active extracts and fractions of the most potent extract from *E. macrophylla*, and chloroquine solution in β-hematin formation assay. The values were reported as Mean ± SD.

phenolic acids, stilbenes, lignans and alkaloids exhibited antiplasmodial activity in different antimalarial assays.[22-37] Based on the results shown in Table 2, in 60% ethyl acetate/n-hexane fraction, steroidal structures were identified as the major active constituents (about 82%). Therefore, it seems that the strong antimalarial activity of this fraction might be related to the presence of steroids derivatives. In the case of 80% ethyl acetate/n-hexane and 100% ethyl acetate fractions, fatty acid, polycyclic aromatic hydrocarbon, steroids and their derivatives were identified by GC-MS analysis. Additionally, in 100% MeOH fraction, the presence of fatty acids, steroids and diterpenoid derivatives were determined. Moreover, based on previous researches, the presence of lipids and other fatty acids in the mixture of active extracts and fractions showed synergistic effects with oleic acid in assay; therefore, the recorded absorbances were higher than the negative control. It was exhibited that the values of IC_{50} and IC_{90} could be reduced by entirely eliminating the fatty acids and purifying the active antimalarial constituents.[14,45] The percentage of fatty acids and their derivatives in 100% MeOH fraction (23.14%) was higher than that in 80% ethyl acetate/n-hexane (5.60%) and 100% ethyl acetate (3.46%) fractions; so the IC_{50} and IC_{90} values of 100% MeOH fraction can be extremely reduced by completely removing of fatty acid derivatives. In consideration of high yields of 100% MeOH fraction (68.18% of DCM extract) as compared to other active fractions (Table 1), more purification of this fraction would be valuable.

Taken all, based on the GC-MS analysis of VLC fractions and previous studies, it seems that steroid derivatives and diterpenes are as the major active antimalarial constituents in DCM extract of rhizomes. Among the seven different polarity fractions, 20% ethyl acetate/n-hexane and 40% ethyl acetate/n-hexane fractions revealed no activity in this assay system, while the last four polar fractions showed the potent activity with close IC_{50} and IC_{90} values (Table 1 and Fig. 1).

Conclusion

From 6 extracts with different polarity of *E. macrophylla*'s aerial parts and rhizomes, the DCM extract of both parts were the most active extract in the cell free *β*-hematin formation assay. The preliminary phytochemical study on the VLC fractions of the most potent part (DCM extract of rhizomes) produced compelling results that led us to focus on purifying the active components of these extracts and investigating further on animal models for in vivo evaluation.

Ethical issues

Not applicable in this study.

Table 2. Chemical composition of VLC fractions of DCM extract from rhizomes

Fraction	Total identified content (%)	Compounds (content %)
Fr.10%	92.47	Linear Aldehyde(10.33%), Alkanes (48.30%), fatty acids and their derivatives(12.13%) , steroids and their derivatives(21.70%)
Fr.20%	*	*
Fr.40%	*	*
Fr.60%	82.32	Steroids (Campesterol (9.03%), Ergosta-5, 22-dien-3-ol, 24-methyl-, (3.beta. 22E)-(6.80%), Clionasterol (66.49%)
Fr.80%	84.39	Fatty acids and their derivatives (5.60%), steroids and their derivatives (25.04%), Linear Alcohol (8.04%), polycyclic aromatic hydrocarbons (45.71%),
Fr.100%	83.6	Fatty acids and their derivatives (3.46%), polycyclic aromatic hydrocarbon (59.46%), steroids and their derivatives (20.68%)
Fr.100%MeOH	97.07	Fatty acids and their derivatives (23.14%), steroids and their derivatives (54.05%), Diterpenoid derivatives (19.88%)

*Not identified.

Research Highlights

What is current knowledge?

√ Plant kingdoms can be potentially rich source of novel antimalarial agents.

√ Resistance to current antimalarial drugs like artemisinin and aminoquinolines has threatened malaria eradication efforts; therefore, several screening programs have been carried out to find new natural sources of antimalarial agents from different plant families.

What is new here?

√ DCM extract from rhizomes of *E. macrophylla* showed antimalarial activity in in vitro β-hematin formation assay.

√ Fractionation of DCM extract and evaluation of them by GC-MS suggested that steroidal compounds may be responsible for antimalarial activity.

Competing interests

Authors declare no competing interests.

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