



Phytochemical and Antimalarial Effects of *Ecballium elaterium* (L.) Rich. Growing in Iran

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Abstract

Background: Malaria is a well-recognized parasitic disease and a serious public health problem worldwide, particularly in tropical and subtropical areas.

Objectives: This study was conducted to investigate the antimalarial properties of extracts with different polarities from the various parts of *Ecballium elaterium* (L.) Rich. (or wild cucumber) as a perennial herbaceous plant growing in Gilan and Azerbaijan provinces of Iran.

Methods: The air-dried and powdered fruits, seeds, and roots of *E. elaterium* were extracted using three solvents with different polarities, n-Hexane (n-Hex), dichloromethane (DCM), and methanol (MeOH). The MeOH extract of roots was subjected to fractionalizing by a C₁₈ Sep-Pak cartridge. All extracts and fractions with different polarities were assessed for their antimalarial activity using the cell-free beta-hematin formation test, and the structural groups of the fractions were identified by Thin-Layer Chromatography (TLC).

Results: According to our results, the MeOH extracts of the plant's roots presented considerable antimalarial effects with an IC₅₀ value of 0.124 ± 0.0002 mg/mL. Bioactivity-guided fractionation of root MeOH extract by solid phase extraction (SPE) afforded six fractions. The 20% fraction showed the most potent antimalarial effect with an IC₅₀ value of 0.167 ± 0.002 mg/mL. Moreover, the three fractions of 80%, 60%, and 100% methanol/water demonstrated considerable antimalarial activities. Phytochemical analysis of potent fractions of *E. elaterium* suggested the presence of flavonoids in 20% and 60% fractions and flavonoids and triterpenoids in 80% and 100% fractions.

Conclusions: According to our primary phytochemical investigation on the six SPE fractions, it is recommended to purify the active constituents of the most effective fractions and investigate their biological effects in animal models.

Keywords: Anti-malaria, *Ecballium elaterium*, TLC, IC₅₀

1. Background

Malaria is a well-known parasitic disease and an important public health problem, particularly in tropical and subtropical areas. Despite many efforts, this infectious disease infected over 200 million cases and caused 438000 deaths worldwide in 2015 (1-3). The disease is transmitted by the bite of the *Plasmodium*-infected female *Anopheles* sp. mosquito, and it is caused by intracellular parasitic protozoa of the genus *Plasmodium* (4).

Moreover, the disease is one of the most prevalent parasitic infections in Southeastern Iran (Sistan and Baluchestan, Hormozgan, and the tropical areas of Kerman province), constituting about 95% of all malaria cases

in the country (5).

Studies on malaria treatment indicate the necessity of introducing new anti-malaria medications due to increasing rates of drug resistance (4). Nowadays, medicinal plants, as invariable resources for new drugs, have been investigated for their antimalarial properties (6). As an ongoing effort to divulge the antimalarial effects of Iranian plants, we here studied the antimalarial properties of the extracts and fractions with different polarities of *Ecballium elaterium* (L.) Rich.

Ecballium elaterium or wild cucumber (family: Cucurbitaceae), which has wide-range traditional usages, is a monotypic weedy species growing by the roadside and

waste grounds from Northern Spain through Southern Europe to the Mediterranean region, Southwest Asia, and North Africa (7, 8). It also grows as a perennial herbaceous plant in Gilan and Azerbaijan provinces of Iran (9). In folk medicine, different parts of the plant are used for treating rhinosinusitis, sinusitis, malaria, psoriasis, rheumatism, constipation, jaundice, otitis, hepatitis, hemorrhoid, epilepsy, and chronic headache and as an emetic and emollient agent (10-15).

Previous reports revealed several pharmaceutical and biological effects for *E. elaterium*, such as antimicrobial (16), anti-inflammatory (17), anti-trypsin (13), anti-insect (18), antioxidant, cytotoxic (19), and hepato-protective (20) activities. On the contrary, there are several studies that introduce *E. elaterium* as a toxic herb, particularly its juice, with poisonous effects against the respiratory, gastrointestinal, and cardiovascular systems (8).

2. Objectives

The present study aimed to evaluate (a) the antimalarial effects of different extracts of the fruits, seeds, and rhizomes of *E. elaterium*, (b) fractionize the most potent extract, (c) characterize the most potent parts, and finally (d) identify their structural groups using the Thin-Layer Chromatography (TLC) method.

3. Methods

3.1. Chemicals

The solvents that were used for extraction and fractionation were bought from Duksan Laboratories (South Korea). Chloroquine diphosphate, Hematin porcine, sodium lauryl sulfate (SLS), Glucose, Magnesium sulfate, Sodium ethanoate, Sodium phosphate dibasic, Potassium chloride, Sodium chloride, Caustic soda, and sodium hydrogen carbonate were obtained from Sigma Aldrich Company (the UK). Oleic acid was purchased from Fluka (India); HCl (Hydrochloric acid) and Dimethyl sulfoxide (DMSO) from Merck (Germany); and C₁₈ Sep-Pak cartridge from Waters (the US).

3.2. Plant Materials

Different parts of *E. elaterium* were collected during July and August 2015 from the Moghan region in Ardabil province, Iran, which has an altitude of 191 m above sea level (48° 20' 75" N, 39° 38' 63" E). A voucher sample (TbZ-FPh.648) was consigned to the Faculty of Pharmacy herbarium, Tabriz University of Medical Sciences, Tabriz, Iran.

3.3. Extraction, Separation, and Identification of Compounds

The dried and crushed fruits, seeds, and roots of *E. elaterium* (150 g) were consecutively extracted by the n-Hex, DCM, and MeOH solvents using the soxhlet system (1000 mL of each solvent).

Considering the significant antimalarial effects of the MeOH root extract, about 2 g of the mentioned extract was fractionalized using a C₁₈ Sep-Pak cartridge and the solid phase extraction (SPE) method, eluting in a MeOH/water mixture gradient (10:90, 20:80, 40:60, 60:40, 80:20, and 100:0). All these nine extracts and six fractions were distinctly concentrated by an evaporator at 45°C.

3.4. Antimalarial Cell-free Assay

The antimalarial activities of the plant extracts and fractions were assessed using the method described by Afshar et al. with some changes (21). Briefly, various concentrations (0.1 – 2 mg/mL in DMSO) of the samples were incubated with hematin (3 mM, 100 μL), oleic acid (10.0 mM, 10 μL), and HCl (1 M, 10 μL). The last volume was added to 1 mL sodium acetate buffer (pH=5) and incubated overnight at 37°C with continuous mild shaking. Afterward, the samples were centrifuged, and the hemozoin pellet was adequately washed and incubated with 2.5% SDS in phosphate buffer at 37°C (15 min) under regular shaking. In the next step, the samples were washed in sodium bicarbonate (0.1 M, 1 mL) until a clear supernatant was obtained; the supernatant was discarded, and the pellets were dissolved in NaOH (0.1 M, 1 mL). Finally, hemozoin content was determined by measuring the absorbance at 400 nm (Cecil spectrophotometer). The outcome was recorded as the percentage of inhibition (I%) of heme crystallization, employing DMSO as a negative control, using the following equation:

$$\text{Inhibition \%} = \left[\frac{A_n - A_s}{A_n} \right] \times 100 \quad (1)$$

where A_n is the absorbance of the negative control, and A_s is the absorbance of test samples. Chloroquine diphosphate was used as the positive control in this assay (22).

3.5. TLC Analysis of Fractions

3.5.1. Preliminary Phytochemical Analysis of Fractions

The main chemical groups were identified using the TLC method on silica gel 60 F254 (Merck, Germany). Ethylacetate: Water: Formic acid: Acetic acid (100:26:11:11) and Chloroform: Methanol: Glacial acetic acid: Water (64:12:32:8) were used as the solvent system. Anisaldehyde sulfuric acid was used as the reagent, and visualization was performed under UV 366 nm after about 10 minutes at 100°C (23, 24). The Libermann- Buchard and Shinoda tests were performed to verify the results of TLC analysis.

3.6. Libermann-Buchard Test

The plant fractions were exposed to small amounts of acetic anhydride. The obtained mixture was boiled and cooled. Then sulfuric acid was added to the mixture from the sides of the test tube. The creation of a deep red color indicated the presence of triterpenoids (25).

3.7. Shinoda Test

Magnesium ribbon was added to the test sample, and then hydrochloric acid was added. The emergence of a crimson red, pink scarlet, or sometimes green to blue color after a few minutes showed the existence of flavonoids (25).

4. Results

Table 1 summarizes the results of the cell-free beta-hematin formation test for three extracts (*E. elaterium* fruits, seeds, and roots) with different polarities and six fractions of the strongest extract (i.e., MeOH root extract).

Using the beta-hematin formation assay, the inhibition concentration (IC_{50%}) and its standard deviation (SD) were determined for each sample (n=3). The IC₅₀ and IC₉₀ values were calculated graphically by plotting the concentration against the inhibition percentage. Chloroquine diphosphate (i.e., with the most potent antimalarial effect) was used as the positive control and DMSO (i.e., without any antimalarial effect) as the negative control. In terms of seed extracts, although the DCM and MeOH extracts showed significant antimalarial activity compared to the control group (P < 0.001 and P < 0.0001, respectively), no antimalarial activity was observed for the n-hex extract (Table 1). Furthermore, the n-hex and DCM fruit extracts showed moderate antimalarial activity, whereas the MeOH extract could not inhibit hemozoin formation (i.e., no or negligible activity). Finally, different root extracts illustrated significant inhibitory activities compared to the control group (P < 0.001).

Among all the extracts, the IC₅₀ of MeOH root extract was the lowest. Moreover, it considerably (P < 0.001) inhibited the transformation of heme to hemozoin compared to the control, as well as the DCM and n-hex root extracts and MeOH seed extract (with a minimum IC₅₀). Hence, MeOH root extract was considered for fractionation. Among the obtained SPE fractions, although all of them revealed antimalarial activity compared to the control group (P < 0.001), the 10% SPE fraction could not notably inhibit hemozoin production.

The results of the TLC analysis of the most potent antimalarial fractions have been shown in Table 2.

5. Discussion

Malaria is an infectious life-threatening ailment caused by the bites of Anopheles mosquitoes. The mosquito (an intracellular parasite of the genus *Plasmodium*) (26) infects the host's erythrocytes and utilizes hemoglobin as a nutrient source by degrading this protein to amino acids, which are used for energy production, growth, and maturation. Previous reports revealed that up to 80% of the host's hemoglobin is degraded by these microorganisms (27). Hemoglobin degradation by protease enzymes releases the heme (ferrous) that is then oxidized to the ferric form. The latest form is toxic to the parasite and can damage biological membranes and suppress enzymes such as proteases. The parasite, which requires a mechanism to detoxify the released heme, has solved this problem by transforming the ferric heme to an insoluble, inactive, and crystalline form named hemozoin (i.e., the malaria-pigment) (28). Thus, inhibiting hemozoin formation can be a potential therapeutic method for this disease.

Scientific reports revealed the efficacy of several medicinal plants (e. g., different species of *Artemisia*, *Cinchona*, *Cryptolepis*, and *Tabebuia* genera) and their secondary metabolites in the treatment of malaria and pointed out their advantages over modern remedies (e.g., low cost, fewer side effects, etc.). For example, chloroquine is a principal natural anti-malaria medication derived from quinine (29).

In this study, nine different extracts of various parts and six fractions of the MeOH root extract of *E. elaterium* were assessed for their antimalarial activities in vitro. Among the different extracts assessed, the MeOH seed and root extracts (with the minimum IC₅₀ of 0.458 ± 0.094 and 0.124 ± 0.0002, respectively) considerably inhibited heme to hemozoin transformation compared to the control (DMSO) (P < 0.001).

Although different studies have noted that the main ingredients of plants such as sesquiterpenoids and triterpenoids (Cucurbitacin E and α-Elaterin) (30), as potent antimalarial agents (31), are accumulated in fruits, we here observed that the antimalarial activity of the MeOH fruit extract was negligible (a high IC₅₀) compared to the MeOH root extract.

According to the obtained results, we performed the fractionation of the extracts that potentially had potent antimalarial components with high polarities using the SPE method on a C₁ Sep-Pak cartridge with solvent combinations with reducing polarities (21). All of the SPE fractions with different concentrations, except for the 10% fraction, inhibited the formation of hemozoin compared to the control group (P < 0.001). Furthermore, among the fractions,

Table 1. The IC_{50%} and IC_{90%} Values of the Extracts and Sep-Pak Fractions of the MeOH Root Extract of *Ecballium elaterium* in the Cell-free Beta-hematin Formation Assay

Different Parts of the <i>Ecballium elaterium</i> / Extracts	IC ₅₀ (mg/mL) ± SD	IC ₉₀ (mg/mL) ± SD
Seed		
n-hex	-	-
DCM	1.338 ± 0.038	2.214 ± 0.069
MeOH	0.458 ± 0.094	2.703 ± 0.063
Fruit		
n-hex	3.518 ± 0.316	4.332 ± 0.487
DCM	3.641 ± 0.173	20.414 ± 1.958
MeOH	-	-
Root		
n-hex	4.645 ± 0.643	6.606 ± 1.191
DCM	2.637.0 ± 0.224	9.078 ± 1.286
MeOH	0.124 ± 0.0002	1.471 ± 0.009
10% Sep-Pak fraction MeOH	176.493 ± 24.518	871.356 ± 130.730
20% Sep-Pak fraction MeOH	0.167 ± 0.002	0.433 ± 0.005
40% Sep-Pak fraction MeOH	2.530 ± 0.203	6.050 ± 1.109
60% Sep-Pak fraction MeOH	0.375 ± 0.048	1.619 ± 0.018
80% Sep-Pak fraction MeOH	0.251 ± 0.002	0.922 ± 0.004
100% Sep-Pak fraction MeOH	0.563 ± 0.007	1.001 ± 0.019
Chloroquine diphosphate	0.014 ± 0.004	0.164 ± 0.006

Table 2. TLC Analysis of the Most Potent Antimalarial Fractions

Fractions	Identified Chemical Groups
Fr.20%	Flavonoids (brown spots)
Fr.60%	Flavonoids (brown zones)
Fr.80%	Flavonoids (brown spots), Triterpenoids (violet black zones)
Fr.100%	Flavonoids (brown spots), Triterpenoids (violet spots)

the 20% SPE fraction obtained the minimum IC₅₀. Other fractions (80%, 60%, and 100%) also were distinguished with significant and potent antimalarial activity (P < 0.001) (Figure 1).

A preliminary phytochemical analysis of the most potent antimalarial fractions was performed using the TLC method. In addition, the Libermann-Buchard and Shinoda tests were applied to confirm TLC analysis results. The findings suggested the presence of flavonoids in the 20% and 60% fractions and flavonoids and triterpenoids in the 80% and 100% fractions.

Previous phytochemical investigations on *E. elaterium* demonstrated that the plant is a rich source of phenolic compounds such as flavonoids, flavanols, tannins, and carotenoids, as well as proteins triterpenoids, and lipids

(32, 33). Cucurbitacin derivatives with triterpenic structures are among the main phytochemicals of *E. elaterium* and have been investigated for their anti-proliferative, anti-cancer, anti-inflammatory, and antimicrobial properties (34, 35).

According to previous studies, flavonoids and triterpenoids can be responsible for potent antimalarial effects (36, 37). For example, Cucurbitacin glycosides from *Datisca glomerata* (C. Presl) Baill showed antiplasmodial activities (38). Moreover, earlier studies showed that flavonoids had potential synergistic anti-malaria effects with artemisinin (the main antimalarial constituent of *Artemisia annua* L.) (39). Considering our findings and those of previous studies, it is necessary to focus on *E. elaterium* pure ingredients and investigate their biological (such as antimalarial) effects.

5.1. Conclusions

Out of the nine extracts with different polarities of *E. elaterium* different parts, the MeOH root extract was the most potent part in terms of the IC₅₀ obtained in the cell-free beta-hematin formation assay. A primary phytochemical analysis on the SPE fractions of the most effective extracts suggested the need for purifying their active con-

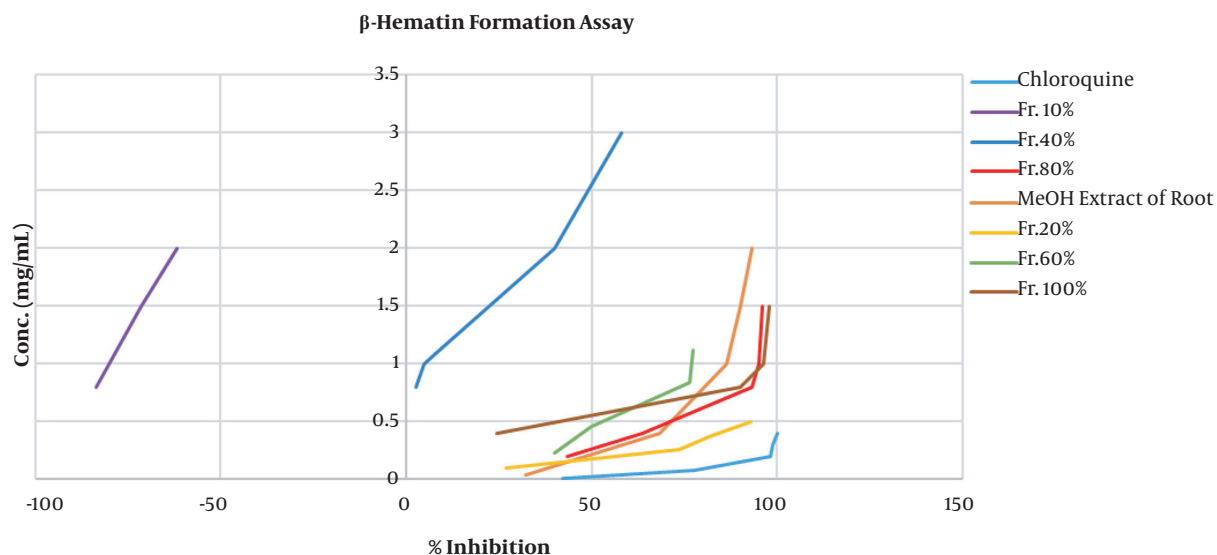


Figure 1. Comparison of the inhibition percentage of heme crystallization between the *E. elaterium* MeOH root extract and its active fractions. Chloroquine diphosphate served as the positive control in the β -hematin formation assay.

stituents and investigating their biological effects in animal models.

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Footnotes

Authors' Contribution: Parina Asgharian and Abbas Delazar designed the study. Zahra Ghalbi, Yaser Sarvari, and Sedigheh Bamdad carried out the experiment and reviewed the literature. Solmaz Asnaashari prepared the manuscript, analyzed the data, and verified the accuracy of the tests.

Conflict of Interests: There is no conflict of interest.

Ethical Approval: The study has been approved by the ethical committee in research of Tabriz University of Medical Sciences (IR.TBZMED.REC.1395.1078).

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