Original Article

Anti-Plasmodial Assessment of Four Different Iranian Propolis Extracts

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Abstract

Background: Eradication of malaria will depend on discovery of new intervention tools such as anti-malarial drugs. Due to the increasing interest in the application of propolis against significant clinical pathogenic agents, the aim of the present investigation was to evaluate the anti-plasmodial effect of Iranian propolis extracts against chloroquine (CQ)-sensitive *Plasmodium falciparum* 3D7 and *Plasmodium berghei* (ANKA strain).

Methods: Crude samples of honeybee (*Apis mellifera*) propolis were collected from four provinces in northern (Kalaleh, Golestan), northeastern (Chenaran, Razavi Khorasan), central (Taleghan, Alborz) and western (Morad Beyg, Hamedan) areas of Iran with different types of flora. The dried propolis samples were extracted with three different solvents, including ethanol 70% (EtOH), ethyl acetate (EA) and dichloromethane (DCM).

Results: All extracts were shown to have *in vitro* anti-plasmodial activity with IC_{50} ranging from 16.263 to 80.012 µg/mL using parasite lactate dehydrogenase (pLDH) assay. The DCM extract of Morad Beyg propolis indicated the highest anti-plasmodial activity (IC_{50}: 16.263 ± 2.910 µg/mL; *P* = 0.027, Kruskal-Wallis *H*-test). The samples were also evaluated in mice for their *in vivo* anti-plasmodial effect. The curative effect against established infection (Rane test) showed that both extracts at all doses (50, 100, and 200 mg/kgBW) produced anti-plasmodial activity against the parasite. Furthermore, using gas chromatography-mass spectrometry (GC-MS), the quantity of flavonoids in DCM and EtOH 70% extracts were found to be 7.42% and 3.10%, respectively.

Conclusion: The potent anti-plasmodial activity of both EtOH 70% and DCM extracts of the propolis of Morad Beyg, Hamedan suggests further analyses of individual components to assess its utilization as anti-malarial drugs.

Keywords: Anti-plasmodial activity, Iran, malaria, *P. berghei* ANKA, *P. falciparum* 3D7, propolis

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Introduction

Human malaria is endemic in 109 countries and prevalent in tropical and sub-tropical regions. According to recent reports, 3.3 billion people are at risk of contracting the infection, of which 1.2 billion are at high risk. In 2013, an estimated 198 million cases of malaria with 755,000 deaths were documented.[1] Since the launch of the Roll Back Malaria Initiative by the WHO in 1998,[2,3] malaria control has augmented in endemic countries to achieve elimination. However, malaria remains the main cause of morbidity and mortality, particularly among pregnant women and infants under the age of 5 years.[2]

Eradication of malaria, which is a long-term goal, is not possible with tools available today, and it will depend on the success of research and development to discover new intervention tools such as anti-malarial drugs and insecticides. The rapid and wide-spread parasite resistance to existing anti-malarial drugs and insecticides could abolish efforts to eliminate the disease and frustrate the hope to eradicate malaria from the entire world[4] as practical vaccines against malaria are not available yet.[5] At present, drug resistance of parasite is widespread and since 1996, no new anti-malarial drug has been introduced into clinical practice. In addition, there has been recently an increase in parasite strains with reduced sensitivity to the newest drugs.[6] Therefore, resistance to the cheap and available anti-malarial agents has become increasingly important and an urgent global problem, demanding the search for more efficient drugs from natural products. Anti-malarial drugs such as quinine and artemisinin originate from plant extracts *Cinchona calisaya*[7] and *Artemisia annua*,[8] respectively. This issue has motivated many researchers to further intensify and accelerate the search for discovery and development of new generations of natural-product-derived anti-malarial drugs for both control and eradication of malaria.

Propolis is a resinous and sticky hive substance collected by honeybees from various parts of plants and buds. It is used for construction and repair of cracks in the bee hive and also preserves the hive from extreme moisture or drought condition, embalms dead invaders and prevents the development and spread of microbial diseases.[9] Propolis is a mixture of resin, essential

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oils and waxes. It contains amino acids, minerals, ethanol, vitamins A, B complex and E, as well as flavonoids.[10] Propolis also displays strong anti-microbial activity[11] and has been used as a chemotherapeutic agent alone or with many medicines and homeopathic products since ancient times. Because of its wide use in folk medicine for medicinal and cosmetic purposes, propolis has been the subject of intense pharmacological and chemical study for almost 30 years.[12] Besides its traditional uses, it has recently gained popularity as a food supplement and is used extensively in foods and beverages in various parts of the world (especially in Japan and Brazil) to improve health and prevent diseases.[11–17]

Numerous studies have shown that propolis has various biological activities, including anti-inflammatory, anti-cancer, anti-oxidant, anti-hepatotoxic and immunostimulating properties.[16,18–29] Moreover, diverse studies have also been reported its biological activity against infectious diseases such as parasitic vaginal infections,[30] facial septic injury[31] and giardiasis,[32] leishmaniasis,[33,34] as well as malaria[35,36] and viral infection.[37] Therefore, propolis is one of the most potent natural antibiotics, and it seems that its therapeutic use does not induce pathogen resistance.[38] The chemical composition of propolis is very complex and may change based on the local flora at the site of collection and also the season of collection.[11,39–43]

Due to the increasing interest in the application of propolis against significant clinical pathogenic agents and on the basis of the folkloric uses of propolis, the aim of the present investigation was to evaluate the anti-malarial activities of the crude extract of four Iranian propolis samples. This investigation has elicited a global search for alternative anti-plasmodial agents to overcome parasite drug resistance as it is expected that propolis may be utilized as an anti-malarial drug.

Methods

Study areas and propolis samples preparation

Crude samples of honey bee (*Apis mellifera*) propolis were collected from four provinces in northern (Golestan), northeastern (Razavi Khorasan), central (Alborz) and western (Hamedan) areas of Iran with different types of flora (Figure 1). Honeybees use the flora surrounding their hives for propolis production; hence, its chemical composition depends on the local flora at the site of collection.[11,40] Therefore, to collect propolis, we selected Taleghan (Alborz Province), Morad Beyg (Hamedan Province), Kalaleh (Golestan Province) and Chenaran (Razavi Khorasan Province) that are covered mostly with *Ferula ovina* Boiss, *Prunus avium L.*, poplar species and *Juniperus* spp, respectively. The crude propolis samples were collected by scraping the propolis sample of the top of the hive using a spatula during August and September 2014. All collected propolis samples were packed into plastic bags and sent to Honeybee Department of Animal Science Research Institute of Iran and stored in a dark place at 4°C.

Extraction and isolation of propolis compounds

To remove the unwanted materials and preserve the active components, we used different extraction methods. In general, 30 g of the frozen propolis was chopped into small pieces and grounded into fine powder using a mortar and pestle. The powdered samples were stored in appropriate containers and kept at 4°C. For extraction, the unwanted bee wax was first removed by mixing 30 g crude propolis extract with 100 mL of n-hexane at a ratio of 3:10 (w/v) (Merck, MA, USA). After 4-day shaking (120 rpm) at 30°C, the hexane solvent was filtered with Whatman 42 filter paper (Sigma-Aldrich Co., St. Louis, MO, USA), and green propolis samples were dried at room temperature. After removing

Figure 1. Map of Iran showing the geographic distribution of Iranian propolis studied samples. **A:** Alborz Province (Taleghan), **H:** Hamedan Province (Morad Beyg), **G:** Golestan Province (Kalaleh), **R-Kh:** Razavi Khorasan Province (Chenaran).

unwanted wax, the dried propolis samples were extracted with three different solvents (Merck, MA, USA), including ethanol 70% (EtOH; polar), ethyl acetate (EA; semi-polar) and dichloromethane (DCM; non-polar). In these three extraction methods, 6 g of propolis hexane extract was mixed in 20 mL of EtOH 70%, or EA or DCM [3:10 (w/v)] on a shaker (120 rpm) in the dark at 30°C for 3 days. Then, the extracted materials were filtered with Whatman 42 filter paper under vacuum. The organic solvent of the filtered extract was removed, and the crude extracts were concentrated and dried under reduced pressure at 50°C, 40°C and 40°C, respectively, by a rotary evaporator. Various concentrations of the test compounds were dissolved in dimethylsulphoxide (DMSO, Sigma-Aldrich Co., St. Louis, MO, USA) at 50 mg/mL and then stored at 4°C for use in anti-plasmodial assay and general cytotoxicity tests.

Effect of propolis on cell viability using MTT (3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay

L929 mouse fibroblast cell line was maintained in RPMI 1640 medium (Gibco-BRL, Pisley, UK) supplemented with 10% FCS in a humidified incubator (37ºC and 5% CO_2). Inhibition of the growth of L929 mouse fibroblast cell line was used as a measure of the toxicity of the propolis extracts using MTT assay. Extracts (50 mg/mL) were prepared by dissolving in DMSO and then serially diluted (25, 50, 100, 200, 400 and 800 µg/mL) in RPMI 1640 to the desired concentrations. The final DMSO concentration did not exceed 1%, which indicates that this amount has no harmful effect on cells. At the time of assay, cells were seeded at 1×10^4 cells/well (100 µL/well) in 96-well plates. Then, the cells cultured in 96-well plates were incubated in a 5% CO_2 humid atmosphere incubator (INC108, Memmert, Germany) at 37°C for 24 h. After incubation, the medium was removed, and the cells were washed with ×1 PBS (phosphate buffered saline sterile, pH 7.4) and incubated in 100 µL of the culture media containing different propolis concentrations in a 5% CO_2 incubator at 37°C for 24 h. Culture medium without propolis and 1% DMSO solution in distilled water were used as controls. After 24 hours of treatment, the medium was removed, and the cells were washed in ×1 PBS (pH 7.4), followed by incubation in 100 µL of RPMI 1640 containing 5 mg/mL MTT in a 5% CO_2 humid atmosphere incubator at 37°C for 4 h. After incubation, the medium was removed, and the cells were washed with ×1 PBS (pH 7.4). To develop the color, 100 µL of isoporopanol was added, and the plate was shaken gently for 10 min. Absorbance was measured on a microplate reader (ELX808, BioTek, USA) at a wavelength of 570 nm. Absorbance from untreated cells was considered as 100% of growth and used for viability calculation. The percentages of viability were calculated based on the following formula: Number of viable cells counted/total cells counted (viable and dead) × 100 = % viable cells. The impact of toxicity was determined by analyzing the selectivity index (SI); the ratios between the 50% L929 cytotoxic concentration and the anti-plasmodial IC_{50} values for each tested extract.

P. falciparum strain and in vitro culture

Laboratory-adapted *P. falciparum* 3D7 [chloroquine (CQ)-sensitive] strain was continuously cultured based on the modified method described before.[44] Briefly, parasites were maintained in continuous culture on human erythrocytes (blood group O^+ was obtained from the Blood Transfusion Organization, Tehran, Iran) in RPMI 1640 medium supplemented with 10% human AB^+ serum, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 25 mM $NaHCO_3$ and 60 µg/mL gentamicin sulfate, pH 7.2. The cultures were incubated in an atmosphere of 91% N_2, 6% CO_2 and 3% O_2 at 37°C. Parasite cultures were synchronized to the ring stage by treatment with 5% D-sorbitol.[45]

In vitro anti-plasmodial assay

Propolis extracts were assessed for anti-plasmodial activity *in vitro* using modified parasite lactate dehydrogenase (pLDH) method as described previously.[46,47] Briefly, crude extracts of propolis were first dissolved in DMSO at concentration of 50 mg/mL, sonicated for 10 min and then diluted in a malaria culture medium to prepare an 8 mg/mL solution. The highest concentration of solvent that the parasites were exposed to was < 1%, indicating no measurable effect on parasite viability. Microtitration techniques were used to measure the activity of samples over a wide range of concentrations (ranging from 6.25 – 400 µg/mL). Chloroquine diphosphate (Sigma Chemical, USA) was dissolved in double distilled water (1000 mg/mL) and served as control in all experiments. All tests were performed in triplicate. Synchronous cultures with parasitemia of 2% and final hematocrit 1.2% were aliquoted into the plates and incubated at 37°C for 48 h. After incubation period, the plates were frozen at -20°C overnight, followed by thawing at room temperature to hemolyze the red blood cells. Parasite growth was determined spectrophotometrically at 650 nm by measuring the activity of the pLDH in control and drug-treated cultures, using a microplate reader (PowerWave 340, BioTek, USA). At the end of incubation, the cultures were resuspended and 20 µL aliquots were removed and added to 100 µL of the Malstat reagent[46,47] in a 96-well microtiter plate. Spectrophotometric assessment of pLDH activity was obtained by adding 25 µL of a solution of 1.9 µM NBT (nitro blue tetrazolium) and 0.24 µM PES (phenazine ethosulphate) to the Malstat reagent. The anti-malarial activity of the test compound was expressed as IC_{50} (mean ± S.D. of the least three separate experiments performed in triplicate). The inhibition of each extract or drug concentration was calculated as compared to the untreated control to obtain the IC_{50} values. These values were then expressed as a percentage of 100% growth value and plotted against corresponding concentrations of the drug, using Gene5 micrroplate data collection and analysis software (BioTek, USA) to generate log-dose response curves.

Evaluation of anti-plasmodial activity on established infection (Rane test)

In vivo anti-plasmodial (schizontocidal) activity of the EtOH 70% and DCM extracts of the Morad Beyg (due to highest SI and *in vitro* anti-plasmodial activity in the present work) was assessed by a curative test (Rane test) using the method described earlier.[48] The mice were purchased from Pasteur Institute of Iran, were housed in an experimental room in standard polypropylene cages and acclimatized for 10 days prior to the experiments. The anti-plasmodial study was carried out in compliance with the "Principles of Laboratory Animal Care" (NIH Publication No. 85; rev. 1985) based on the approval of the Ethics Committee of Pasteur Institute of Iran. Female BALB/c mice, weighing 18 – 20 g were infected by interaperitoneal (i.p.) inoculation of 10^7 infected erythrocytes with CQ-sensitive *Plasmodium berghei* (ANKA strain) in a saline suspension of 0.2 mL on the first day (D0) of the experiment and left untreated for 72 h. Three days later, the

infected mice were weighed and randomized into nine groups of five mice each. The first treatment took place 72 h after the mice were infected and treated daily for five days. Groups 1 and 2 were given PBS and DMSO (15%) as negative controls. Groups 3 mice received 25 mg CQ /kg body weight daily i.p. for 5 days as positive control. Groups 4, 5 and 6 were treated with 50, 100 and 200 mg EtOH 70% extract/kg/day i.p., respectively. Groups 7, 8 and 9 were given with 50, 100 and 200 mg DCM extract/kg/day i.p., respectively. On each day of treatment, blood from the tail of each mouse was smeared on a microscope slide to make thin and thick films, stained with 10% Giemsa in phosphate buffer, pH 7.2 for 15 min and examined under microscope at 100× to monitor the parasitemia level. The percentage parasitemia was determined by counting the parasitized red blood cells on at least 3,000 red blood cells. The suppression percentage of parasitemia for each extract was calculated by comparing the parasitemia present in the infected control with those of treated mice (% suppression = parasitemia in negative control (PBS group) - parasitemia in treated group/ parasitemia in negative control ×100). In addition, mortality in mice was followed up to 28 days post treatment (day 31 post-infection), and the parasitemia of the survivors was evaluated.

Statistical analysis

Non-parametric Kruskal-Wallis *H*-test was performed to compare the mean level of IC_{50} (*in vitro*) and mean level of parasitemia (*in vivo* test) of all tested groups; however, comparison between two tested groups was done by Mann–Whitney *U*-test. Bonferroni adjustment was used for correction of *P*-value of multiple comparison tests. In each *in vitro* anti-plasmodial assay, 50% of parasite growth (IC_{50}) was determined; the results were expressed as means ± S.D. Furthermore, the differences between median survival times of all treated groups were reported by log-rank test using the Kaplan-Meier (KM) method. In log-rank analysis, due to low sample size in each group (n = 15), all treated groups with different doses (50, 100 and 200 mg/kg body weight) were only compared with negative control groups (×1 PBS and DMSO, n = 10).

Data was analyzed using SPSS 20.0 software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). *P*-values < 0.05 were considered statistically significant in all tests.

Gas chromatography mass spectrometry (GC-MS) analysis

The chemical compositions of the EtOH 70% and DCM extracts of the most active propolis in the present study (Morad Beyg from Hamedan Province) were characterized by high-resolution GC-MS. Briefly, the dry propolis samples (5 mg) were mixed to 250 µL pyridine (anhydrous, 99.8 %; Sigma, Saint Louis, Missouri, USA) and 500 µL bis-(trimethylsilyl) trifluoroacetamide (BSTFA), including 1% trimethylchlorosilane (TMCS) (Sigma, St. Louis, Missouri, USA) in a sealed glass tube at 100°C for 30 min to produce trimethylsilyl (TMS) derivatives for gas chromatography as described previously.[49] Then, 1 µL of prepared samples was injected and analyzed by GC-MS. We used an Agilent 6890N gas chromatograph equipped with a split/splitless injector, an Agilent 5975C mass selective detector (MSD), and an auto-sampler Combi PAL (CTC analytics, Switzerland). The mass spectrometer (MS) was operated in the electron ionization (EI) mode (70 eV). Helium (99.999%) was employed as a carrier gas and its flow rate was adjusted to 1 $mL.min^{-1}$. Chromatographic separation of chemical compositions was performed on a GC capillary column HP-5MS (30 m × 250 µm ID and film thickness of 0.25 µm) (J&W Scientific, USA). The initial temperature of the column was set at 40°C and held for 2 min, and then increased by 5°C min^{-1} to 150°C and maintained for 3 min. Finally, it was increased by 20°C min^{-1} to 280°C and held for 10 min. The injector temperature was adjusted at 250°C and desorption process was carried out in the splitless mode for 5min. The temperature of GC-MS interface, ion source and quadrupole were set to 280, 230, and 150°C, respectively. The MS was operated in the scan mode, and MS scan range was 40 – 500 atomic mass units (AMU). The chromatograms showed a group of peaks identified by comparing their retention time and mass spectra. Their mass spectral patterns identified in the Wiley Mass Spectral library were installed on the GC-MS linked computer.

Results

Activity of propolis extracts against *P. falciparum*

The *in vitro* anti-plasmodial effects of the four Iranian propolis extracts against CQ-sensitive *P. falciparum* 3D7 are shown in Table 1 and Figure 2. Three different extracts of propolis (EtOH 70%, EA and DCM) showed anti-plasmodial activity with an IC_{50} ranging from 16.263 to 80.012 µg/mL (Table 1). The DCM extract of Morad Beyg (Hamedan) propolis showed the highest anti-plasmodial activity, with an IC_{50} value of 16.263 µg/ mL against *P. falciparum* 3D7. On the other hand, the EtOH 70% extract of Chenaran (Khorasan Razavi) revealed the lowest anti-plasmodial activity (IC_{50} = 80.012 µg/mL), while CQ as positive control showed IC_{50} values of 0.16 µg/mL for *P. falciparum* 3D7 line. Moreover, comparison of anti-plasmodial activity of

Table 1. Comparison of *in vitro* anti-plasmodial activity of the Iranian propolis extracts obtained from different study areas against *P. falciparum* 3D7 strain.

Study area	IC50 ± SD (µg/mL)						
	SI	Ethanol 70%	SI	Ethyl acetate	SI	Dichloromethane	*P-value*
Taleghan (Alborz)	7.01	47.074 ± 10.142	6.65	45.784 ± 6.776	9.37	35.213 ± 11.866	0.430
Chenaran (Razavi Khorasan)	3.74	80.012 ± 3.912	7.01	57.000 ± 2.645	10.88	30.308 ± 0.804	0.027*
Kalaleh (Golestan)	5.02	53.770 ± 3.673	5.62	49.797 ± 3.615	8.28	34.999 ± 4.352	0.051
Morad Beyg (Hamedan)	10.71	27.066 ± 2.271	7.75	37.651 ± 7.239	17.83	16.263 ± 2.910	0.027*
P-value	- - -	0.034*	- - -	0.022*	- - -	0.053	- - -

SI: Selectivity Index; *P*-values were calculated using Kruskal Wallis H. $P < 0.05$ was considered statistically significant and shown by star. IC_{50} of the CQ for 3D7 strain was 0.16 ± 0.1 µg/mL.

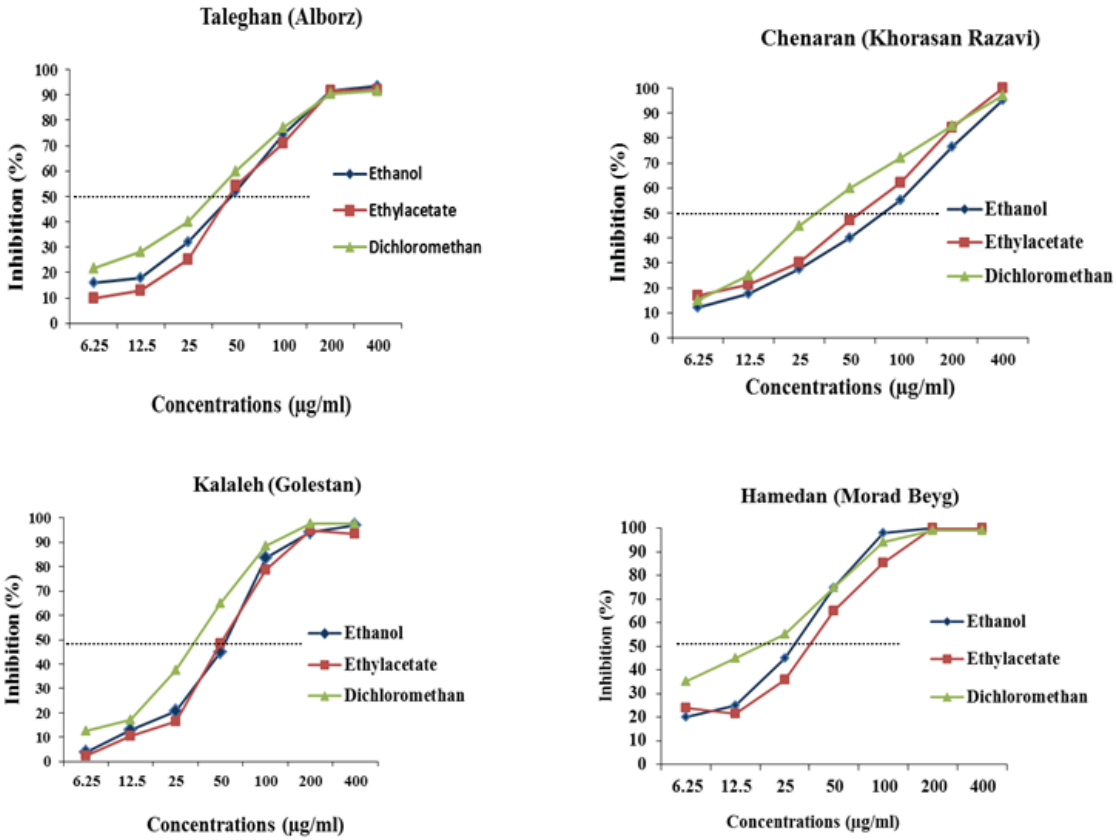

Figure 2. Concentration effectiveness of propolis extracts collected from different study areas against *P. falciparum* 3D7 strains. Seven concentrations of extracts (6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL) were used. Values are presented as mean of three independent experiments. The horizontal dashed lines represent the 50% inhibition (IC_{50}). An IC_{50} value of 0.16 ± 0.1 was obtained for CQ as positive control.

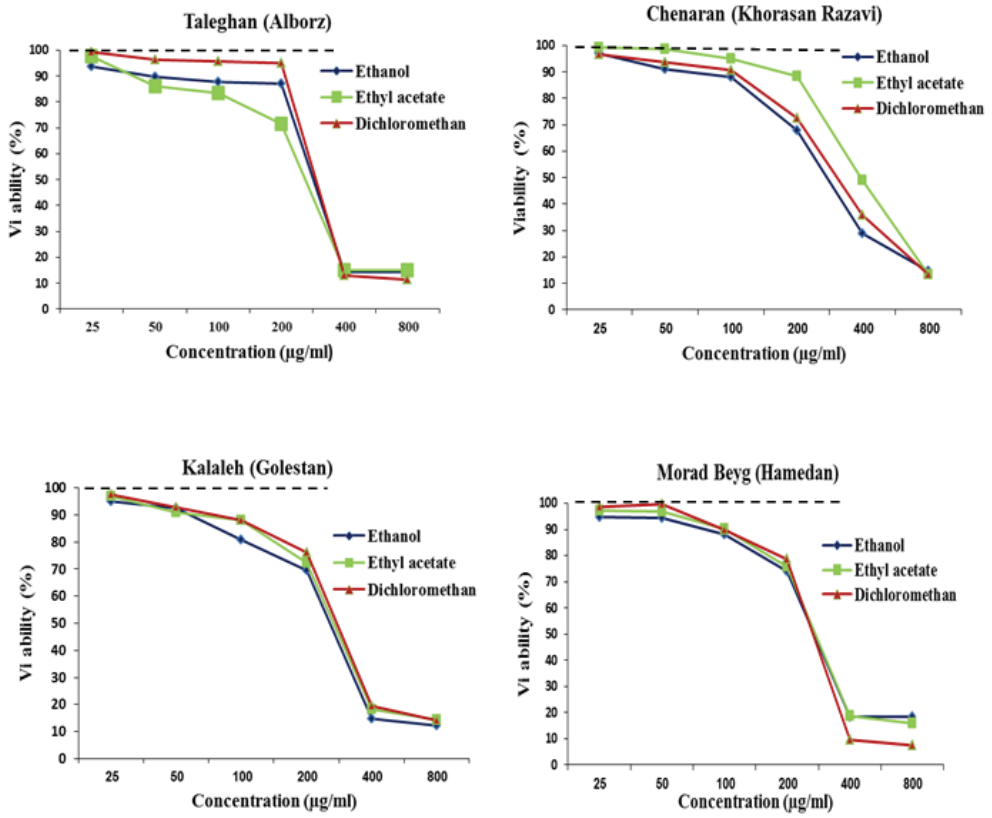

Figure 3. Viability of L929 fibroblast cells in different concentrations of propolis extracts collected from different study areas. Six concentrations of extracts (25, 50, 100, 200, 400 and 800 µg/mL) were used. Values are presented as mean of three repeats. The horizontal dashed lines represent 100% viability of cells in the absence of propolis extracts (control).

Table 2. The *in vitro* anti-plasmodial activities of global propolis

Origin of propolis	Flora	*Propolis color*	*P. falciparum strain*	*In vitro* assay	Solvent Extraction	IC_{50} ± SD (µg/mL)	Reference
Iran			3D7(S)	pLDH			
Taleghan	*Ferula ovina* Boiss.	Dark green	- - -	- - -	Ethanol 70% Ethyl acetate Dichloromethane	47.074 ± 10.142 45.784 ± 6.776 35.213 ± 11.866	Current study
Chenaran	*Juniperus spp.*	Green	- - -	- - -	Ethanol 70% Ethyl acetate Dichloromethane	80.012 ± 3.912 57.000 ± 2.645 30.308 ± 0.804	
Kalaleh	Poplar *spp.*	Dark green	- - -	- - -	Ethanol 70% Ethyl acetate Dichloromethane	53.770 ± 3.673 49.797 ± 3.615 34.999 ± 4.352	
Morad Beyg	*Prunusavium L*	Green	- - -	- - -	Ethanol 70% Ethyl acetate Dichloromethane	27.066 ± 2.271 37.651 ± 7.239 16.263 ± 2.910	
Control (CQ)	-					0.16 ± 0.1	
Brazil	NA	Green	D6 (S) W2 (R)	pLDH	Ethanol 70%	20 13	Filho, et al. 2009
	NA	Green	D6 (S) W2 (R)	pLDH	Ethanol 70%	20 13	
Control (CQ)	- - -	- - -	D6 (S) W2 (R)	- - -	- - -	0.018 0.176	
Control (Art)	- - -	- - -	D6 (S) W2 (R)	- - -	- - -	0.014 0.008	
Indonesia	NA	NA		SYBR Green I	Ethanol 70%		Syamsudin, et al. 2011
Lawang	- - -	- - -	D6 (S) W2 (R)	- - -	- - -	37.34 ± 1.23 89.45 ± 2.13	
Sukabumi	- - -	- - -	D6 (S) W2 (R)	- - -	- - -	215.23 ± 2.32 453.23 ± 3.45	
Batang	- - -	- - -	D6 (S) W2 (R)	- - -	- - -	189.98 ± 1.34 356.89 ± 2.34	
Control (CQ)	- - -	- - -	D6 (S) W2 (R)	- - -	- - -	0.0123 ± 0.11 1.67 ± 0.12	
Cuba	NA		Ghana (S)	pLDH	Methanol 100%		Monzote, et al. 2012
JardínBotánico		Brown	- - -	- - -	- - -	11.6 ± 5.4	
Buey Arriba		Brown	- - -	- - -	- - -	0.2 ± 0.1	
Buey Arriba		Brown	- - -	- - -	- - -	10.8 ± 5.2	
Imías		Brown	- - -	- - -	- - -	12.5 ± 6.9	
Puerto Padre		Brown	- - -	- - -	- - -	9.7 ± 3.6	
Salvador		Brown	- - -	- - -	- - -	6.0 ± 3.9	
Cabo de San Antonio		Red	- - -	- - -	- - -	1.8 ± 1.1	
Manicaragua		Red	- - -	- - -	- - -	5.1 ± 1.2	
La Coloma		Red	- - -	- - -	- - -	1.5 ± 0.1	
Güanes		Red	- - -	- - -	- - -	1.2 ± 1.2	
Jagüey Grande		Red	- - -	- - -	- - -	2.3 ± 1.3	
Villa Clara		Red	- - -	- - -	- - -	3.6 ± 2.8	
Nueva Paz		Red	- - -	- - -	- - -	5.6 ± 1.2	
Ciego de Ávila		Red	- - -	- - -	- - -	2.5 ± 1.3	
Artemisa		Red	- - -	- - -	- - -	6.4 ± 3.4	
Candelaria		Yellow	- - -	- - -	- - -	0.2 ± 0.1	
Bahía Honda		Yellow	- - -	- - -	- - -	6.4 ± 0.6	
Unión de Reyes		Yellow	- - -	- - -	- - -	1.7 ± 0.6	
Unión de Reyes		Yellow	- - -	- - -	- - -	1.1 ± 1.5	
Báguanos		Yellow	- - -	- - -	- - -	0.2 ± 0	
Control (CQ)		- - -	- - -	- - -	- - -	0.3 ± 0.1	
Thailand (QA)	NA	NA	K1 (R)	Microculture radioisotope	Water and ethanol	4.48	Kaewmuangmoon, et al. 2012
Portugal		NA	GHA (S)	pLDH	Ethanol 80%	30.1 ± 4.1	Falcao, et al. 2014
North-eastern Center	*Populuscanadensis and Cistusladanifer L.*	- - -	- - -	- - -	- - -	8.8 ± 1.8	
Populuscanadensis (male)	- - -	- - -	- - -	- - -	- - -	10.9 ± 2.1	
Populuscanadensis (female)	- - -	- - -	- - -	- - -	- - -	28.5 ± 3.8	
CistusladaniferL	- - -	- - -	- - -	- - -	- - -	17.0 ± 2.5	
Control (CQ)	- - -	- - -	- - -	- - -	- - -	0.04 ± 0.01	

NA: not available; Art: Artemisinin; CQ: Chloroquine; S: CQ sensitive; R: CQ resistant; QA: Control (Art); pLDH: parasite lactate dehydrogenase

Iranian propolis extracted with three different solvents revealed significant differences for Chenaran (P = 0.027) and Morad Beyg (P = 0.027) (Table 1). All four tested extracts indicated a concentration-dependent growth inhibition of the *P. falciparum* 3D7 parasite (Figure 2), and this result again confirmed the highest activity of DCM extract of Morad Beyg (Hamedan) at lowest concentration of 16.263 µg/mL.

Toxicity of propolis against mammalian cells (L929)

We assessed the viability of the L929 fibroblast cells in the presence of different concentrations of EtOH 70%, EA and DCM propolis extracts at 25 to 800 µg/mL, and all four examined propolis extracts did not show cytotoxicity in ≤ 200 µg/mL concentration (~70% viability, Figure 3). However, the viability at 400 to 800 µg/mL was reduced to < 50% for Chenaran and < 20 for Taleghan, Kalaleh, and Morad Beyg propolis extracts (Figure 3).

In vivo anti-plasmodial activity on established infection

The EtOH 70% and DCM extracts of Morad Beyg propolis produced dose-dependent and significant decrease in parasite counts in infected mice (EtOH 70%: D7, $P < 0.0001$; D14, $P = 0.002$ and DCM: D7, $P = 0.009$; D14: $P = 0.002$) as compared to the control group (PBS). On day 7 (D7) post infection, there was a significant difference among the mean of the parasitemia in mice group receiving 100 and 200 mg/kg body weight of EtOH 70% extract ($P = 0.009$). No significant difference was observed between the mean parasitemia of mice groups that received 100 and 200 mg/kg body weight of DCM extract ($P = 0.754$). Comparing the mice group receiving 50 mg/kg body weight of DCM and EtOH 70% extracts, the mean parasitemia was significantly different ($P = 0.009$). On day 14 (D14) post infection, the mean suppression of parasitemia for EtOH 70% extract was 65.1%, 66% and 71% and for DCM extract was 59.3%, 62% and 65% at the doses of 50, 100 and 200 mg extract/kg body weight, respectively (Table 4). However, CQ (25 mg/kg) produced 100% suppression. All mice that received ×1 PBS (negative control) or 50, 100 and 200 mg/kg of both EtOH 70% and DCM extracts of the Morad Beyg propolis died before completion of the experiment (day 31). In this regard, the median survival time of the mice which received 50, 100 and 200 mg/kg of the EtOH 70% extract of the Morad Beyg propolis, was 26 days, and for DCM was 25, 26, and 26, respectively (Table 4). The comparison of survival times of treated groups with control mice groups (×1 PBS and DMSO) revealed a prolonged survival time in the groups treated with EtOH 70% ($P < 0.0001$) and DCM ($P = 0.0001$) extracts (Table 4).

Chemical composition of Morad Beyg propolis

In this research, the chemical composition of two active extracts with highest anti-plasmodial activity (EtOH 70% and DCM extracts of Morad Beyg propolis) was analyzed by GC-MS. The results showed the complex composition of samples as listed in Table 3. The main chemical components of samples are given according to the % area and were classified in five groups (fatty acids and their esters, flavonoids, chalcones, cinnamates and other compounds). In EtOH 70% extract, the high quantity identified compounds were different fatty acids and their esters, including linoleic acid, TMS (7.01%) and palmitic acid, TMS (6.49%). Also, 3,5,7-tris (trimethylsilyloxy) flavones (3.10%), chalcone derivatives [chalcone, 2',4',6'- tris (trimethylsilyloxy) (0.59%) and pinostrobin chalcone (0.31 %)], and different cinnamic acid derivatives [caffeic acid, tris-TMS (1.31%); ferulic acid, di-TMS (0.67%); isoferulic acid, di-TMS (0.43%) and p-coumaric acid, di-TMS (0.33%)] were identified (Table 3). Regarding the DCM extract of Morad Beyg green propolis, the highest quantity compound was fatty acids [stearic acid (9.07%) and palmitic acid (8.42%)] and flavonoids [pinocembrin (4.26%), tectochrysin (3.23%) and 4,5-dihydroxy-7-methoxyflavanone (0.71%)]. Other compounds such as dibutyl phthalate, heptacosane, hexadecane and octadecane were identified in both extracts with different quantities (Table 3). Also, pinostrobin chalcone was detected in both EtOH 70 % (0.31%) and DCM (3.95%) extracts with different quantities. Interestingly, the quantities of flavonoids in DCM and EtOH 70 % extracts were 8.2% and 3.10%, respectively (Table 3).

Discussion

The drug resistance developed by malaria parasites is a big challenge for malaria control, elimination and eradication campaigns. Therefore, the search for more effective anti-malarial agents from natural products is highly required as in many malaria-endemic countries of the world, natural and traditional products (plants and insects/products) are commonly used.[50] The present study, for the first time, has attempted to conduct a comparative study on the anti-plasmodial activity of four different samples of Iranian propolis. The reason for this investigation was raised by the previous anti-malarial activities of propolis from Cuba,[36] Brazil,[51] Indonesia,[35] Thailand,[52] and Portugal.[53] For the purpose of this work and in accordance with WHO guidelines,[54] anti-malarial activity of the any extract was classified as highly active ($IC_{50} < 5$ µg/mL), promising activity ($IC_{50} = 5 – 15$ µg/mL), and moderate activity ($IC_{50} = 15 – 50$ µg/mL), while extracts with $IC_{50} > 50$ µg/mL were considered to be inactive. Therefore, anti-plasmodial activities of the Iranian propolis extracts are qualified as "active" when IC_{50} is ≤ 50 µg/ml and extracts with an activity beyond this range were considered "inactive".

The chemical composition of propolis is highly variable and complex, depending strongly on the vegetation available at the site of collection. In general, propolis is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen and other substances.[55] Therefore, EtOH 70% has been the most commonly used solvent to obtain low wax propolis extract rich in biologically active compounds.[35,51–53] Based on this fact, in this investigation, the Iranian green propolis was collected from four distinct geographical areas with different types of flora. EtOH 70 % was used as solvent for extraction of biologically active compounds of all propolis samples. The pLDH assay showed moderate anti-plasmodial activity concerning propolis of Taleghan ($IC_{50} = 47.074 \pm 10.142$ µg/mL, SI = 7.01) and Morad Beyg ($IC_{50} = 27.066 \pm 2.271$ µg/mL, SI = 10.71) with mostly *Ferula ovina* Boiss. However, no activity was found for propolis of Chenaran (IC_{50} values of ≥ 50 µg/mL, SI = 3.74) and Kalaleh (IC_{50} values of ≥50 µg/mL, SI = 5.02) with local flora of the *Juniperus* spp. and poplar species, respectively. Therefore, in the second step, anti-plasmodial activity of the non-ethanolic (poorly soluble compounds in water and oil substances) extracts of propolis was investigated using EA (semi-polar) and DCM (non-polar) as solvents. Surprisingly, the DCM extracts of Morad Beyg propolis showed 1.6 times more anti-plasmodial activity than

Table 3. Chemical characterization of two different extracts of Morad Beyg (Hamedan Province, Iran) propolis by GC-MS

Class of compounds	EtOH 70% extract (area%)	DCM extract (area%)
Fatty acids and their esters	Linoleic acid -TMS (7.01); Palmitic acid, TMS (6.49); Oleic acid, TMS (0.5); Pentadecanoic acid (0.43); Nephrosteranic acid (0.97) ; Oleic acid, tri TMS (0.5); Eicosanoic acid, TMS (0.21); Behenic acid, TMS (0.35); Margaric acid (0.19)	Stearic acid (9.07); Palmitic acid (8.42); Palmitic acid, TMS (1.03); Oleic acid (2.34); Oleic acid, TMS (1.24); Pentadecanoic acid (0.43); Methyl stearate (0.71); Myristic acid (0.66); Methyl palmitate (0.46); 7-Methyl-Z-tetradecen-1-ol acetate (0.2); Margaric acid (0.7)
Flavonoids	3,5,7-tris(trimethylsilyloxy)flavone(3.10)	Pinocembrin (4.26); Tectochrysin (3.23); 4',5-Dihydroxy-7-methoxyflavanone (0.71)
Chalcones	Pinostrobin chalcone (0.31); 2',4',6'-tris(trimethylsilyloxy)chalcone (0.59)	Pinostrobin chalcone (3.95)
Cinnamates	Caffeic acid, tri-TMS (1.31); Isoferulic acid, di-TMS (0.43); p-Coumaric acid, di- TMS (0.33); Ferulic acid, di-TMS (0.67); Cinnamic acid, 3,4-bis(trimethylsiloxy)-, methyl ester (0.1)	- - -
Other compounds	Dibutyl phthalate (0.34); Heptacosane (0.11); 5'-hydroxy-5-methyl-3,4-benzopyrrolizidine (0.67); Glycolic acid, di -TMS (0.1); Hexadecane (0.12); n-Tetradecane (0.1); Malic acid, tri-TMS (0.2); 2-(5-acetyl-2-thienyl)-1,4-naphthoquinone (0.15); Octadecane (0.23); Octamethyltrisiloxane (2.26); D-Glucose, 2,3,4,5,6-pentakis-O-(TMS)- (2.33); Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)- (0.18); Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl) (0.38); 2-(2',4'-dichlorophenoxy) phenylacetic acid (2.33); D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)- (0.94); Androstan-17-one, 5,6-epoxy-3-fluoro-, (3.alpha.,5.alpha.,6.alpha.) (1.88); Pentacosane (0.21); Acetamide, N-[2-(1,3-dioxolo[4,5-g]quinoline (0.22); Stigmast-22-en-3-ol, 4-methyl- (3.alpha.,4.alpha.) (1.93); 12-Azabicyclo[9.2.2]pentadeca-1(14),11(15)-dien-13-one (1.62); Trimethylsilyl ether of glycerol (1.82); Lactic acid, di- TMS (0.44); Succinic acid, di-TMS (0.43); Thiocolchicine (0.4); Oreophilin (0.35); Tricosane (0.25); Malic acid, tri-TMS (0.2); Heneicosane (0.13); +/-.-2-Methoxy-3,8-dioxocephalotax-1-ene (0.23); Methyl-19-norisoanticopalate (deuterate)(0.28); alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl) (4.80); Dimethyl 1,3,5,6-tetramethyl-[1,3-(13C2)]bicyclo[5.5..0]dodeca-1,3,5,6,8,10-hexaene-9,10-dicarboxylate (2.13); Cholest-7-en-3-ol-15-one, 14-methyl- (0.64); 3',4'-dihydro-2'-(morpholin-4-yl)-5',7'-dinitrospiro[cyclopentane-1,3'-quinazoline] (0.83)	Dibutyl phthalate (1.83); Heptacosane (1.90); beta.-Eudesmol (0.77); Docosane (0.39); Agarospirol (0.24); Hexadecane (0.17); Tetradecane (0.38); 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1E,3a.alpha.,7a.beta.)- (0.34); Octadecane (0.23); v1-Octadecene (0.68); Thebaine (0.34); Eicosane (0.85); Diploptene (0.21);

The compounds common to EtOH 70% and DCM extraction of Morad Beyg propolis are highlighted in grey color; TMS: trimethylsilyl

Table 4. Mean parasitemic levels, suppression and the median survival time of established *P. berghei* infection during curative test.

Treatment	DCM						EtOH 70%					
	% Parasitemia (Mean ± SD)			% Suppression		Survival time (day) (Median)	% Parasitemia (Mean ± SD)			% Suppression		Survival time (day) (Median)
	D7	D14	D21	D7	D14		D7	D14	D21	D7	D14	
DMSO 15%	30.38 ± 1.56	66.34 ± 2.31	- - -	- - -	- - -	14	30.38 ± 1.56	66.34 ± 2.31	- - -	- - -	- - -	14
X1PBS	29.52 ± 2.23	65.49 ± 1.54	- - -	- - -	- - -	14	29.52 ± 2.23	65.49 ± 1.54	- - -	- - -	- - -	14
50 mg/kg body weight	13.96 ± 1.55*	26.65 ± 0.73*	54.95 ± 0.98	52.7	59.3	25	16.62 ± 0.49*	22.85 ± 0.69*	45.53 ± 1.77	43.7	65.1	26
100 mg/kg body weight	12.78 ± 2.64*	24.88 ± 0.39*	51.1 ± 1.32	56.7	62	26	13.81 ± 3.55*	22.26 ± 6.95*	42.62 ± 2.79	53.2	66	26
200 mg/kg body weight	12.75 ± 1.66*	22.92 ± 0.68*	48.12 ± 1.27	56.8	65	26	11.04 ± 0.49*	18.99 ± 1.26*	41.07 ± 2.5	62.6	71	26
CQ	- - -	- - -	- - -	100	100	No death	- - -	- - -	- - -	100	100	No death

* Bonferroni corrected *P*-value < 0.05/3 = 0.009 [to compare the parasitemia of the test groups with control (×1 PBS)]

EtOH 70% extract, indicating that different compounds or their quantities in those extracts might have anti-plasmodial activity. To evaluate the *in vivo* anti-plasmodial activity of the EtOH 70% and DCM extracts of Morad Beyg propolis, both extracts were used against *P. berghei* infected mice at a safe dose using the curative test. The results demonstrated potential anti-plasmodial activity for both extracts. Rane's test used in this investigation relies on the ability of *P. berghei* to kill the recipient mouse within six days after standard inoculation. However, if infected mice are treated with anti-malarial drugs and survive beyond 12 days, the drug is considered to have anti-malarial activity. Based on this assumption, median survival time was prolonged (about 25 days post infection) in the entire groups treated with both extracts compared to the control. This result might support the benefit of using Morad Beyg propolis for managing uncomplicated *falciparum* malaria.

Interestingly, this propolis with potential anti-plasmodial activity was collected from *Prunus avium* in Morad Beyg, Hamedan Province of Iran, with mostly sweet cherry (*Prunus avium*) plantations. This plant is an important crop in the world, and mostly cultivated in temperate zones, including around the Caspian Sea, Black Sea and different areas in Europe and Western Asian countries[56] with anti-bacterial activity against *Escherichia coli* and mostly used for treatment of bladder infection in traditional medicine.[57] Besides, in temperate zone of Morad Beyg, other possible sources of propolis could be bud exudates of different *Populus* spp. In the previous studies, this propolis was reported to have a microbicidal activity that could be associated with the presence of flavonoids and derivatives of caffeic acid.[12,58–60]

Moreover, the Chenaran propolis (EtOH 70% extract) collected from *Junipers* species showed the lowest anti-plasmodial activity while DCM extracts had moderate activity. It should be noted that *Junipers* species have been reported as medicinal plants to treat malabsorption syndrome and gum bleeding.[61–63] Also, Juniper essential oil and hexane and methanol extracts demonstrated anti-microbial activities against Gram-positive and Gram-negative bacteria species.[64,65] This variability in anti-plasmodial activity could depend on different constituents of propolis collected by the honeybee from plants of different geographic locations.[66]

The dark green propolis collected from Kalaleh, Gorgan Province of Iran, was harvested from poplar plants. This plant is also found in temperate zones, including Asia, Europe, and North America as well as non-tropical regions of New Zealand and Africa, which has been reported to have high amounts of flavones, falvanones and low phenolic components.[11,67–69] However, the EtOH 70% and EA extracts of the Kalaleh propolis showed no anti-plasmodial activity but the DCM extract displayed moderate activity, indicating that different active components of DCM in comparison with EtOH 70% extract might be involved in biological activity.

Moderate anti-plasmodial activity was observed for the DCM extract of Taleghan propolis that might derive from *Ferula* species. This finding was similar to a previous study that reported propolis obtained from *Ferula* species in Isfahan Province, Iran has anti-bacterial activity.[70] Indeed, the *Ferula* species are distributed in Central Asia, Northern Africa and the Mediterranean region that are used in traditional medicine, with different anti-bacterial, anti-fungal, anti-viral, anti-mycobacterial, anti-protozoal, anti-oxidant and anti-cancer activities.[71–73]

Furthermore, to define the chemical composition of two active extracts with high anti-malarial activity, we analyzed the EtOH 70% and DCM extracts of Morad Beyg propolis by GC-MS, and the results showed differences in their chemical composition. In the EtOH 70% extract, 3,5,7-tris (trimethylsilyloxy) flavones, cinnamate and chalcone derivatives were identified in Morad Beyg propolis, and both compounds have been previously reported to show anti-cancer, anti-microbial, anti-oxidant, anti-malarial and anti-protozoal activities.[74–77] Interestingly, it has been previously shown that chalcone is an intermediate of flavonoids biosynthesis in natural sources, and flavonoids are a large group of polyphenolic compounds with a benzopyran-4-one structure that have various biological activities, such as anti-bacterial, anti-cancer, anti-viral, anti-inflammatory and hepatoprotective activities.[29,78–80]

Regarding the DCM extract of Morad Beyg propolis, the palmitic acid (8.42%) and stearic acid (9.07%) had the highest quantities, and these two fatty acids have been reported to have anti-bacterial, anti-fungal and anti-tumor activities.[81–83] In contrast to EtOH 70%, the DCM extract showed notable quantities of flavonoids, such as pinocembrin (4.26%), tectochrysin (3.23%), 4',5-Dihydroxy-7-methoxyflavanone (0.71%) corresponding to 8.2% of the total identified area, as compared to the low content of 3,5,7-tris trimethylsilyloxy, flavones (3.10%) in EtOH 70% extract of Morad Beyg propolis. Therefore, the anti-plasmodial activity of DCM of Morad Beyg propolis could be associated with these flavonoids. In addition, similar to EtOH 70%, the DCM extract had chalcone (pionostrobin chalcone), which is the precursor of flavonoids biosynthesis in natural sources and supports the anti-plasmodial activity of DCM of Morad Beyg propolis. Another notable compound common to both EtOH and DCM is dibutyl phthalate with anti-microbial activity[84] that has areas of 0.34% and 1.83%, respectively.

In this study, for the first time, the total EtOH, EA and DCM extracts of Iranian popolis were assessed for anti-plasmodial activity. The level of anti-plasmodial activity of the EtOH 70% extract of Iranian propolis was in contrast with the highest activity reported from ethanolic or methanolic extraction of propolis from Brazil,[51] Cuba,[36] Thailand[52] and Portugal.[53] However, the moderate to no anti-plasmodial activity of the Iranian propolis was similar to Indonesian propolis[35] (Table 2). Consequently, distinction of anti-plasmodial activities of four types of Iranian propolis may be related to the difference in chemical composition that honeybees collect from various plants at different geographical locations and climates.[66,85,86] These results provide more data to support that the biological activity of propolis depends on the local plant source and flora.[66,87]

In summary, the present results showed differences between the anti-plasmodial activities of four types of Iranian propolis, suggesting that the Iranian propolis could contain several different bioactive compounds based on the plant source and local flora. In addition, potent *in vitro* and *in vivo* anti-plasmodial activity was found for EtOH 70% and DMC extracts of the propolis of Morad Beyg, Hamedan with the presence of high amounts of flavonoids and chalcone. Therefore, in the next step, it is necessary to analyze anti-plasmodial activity of individual components, such as the flavonoids, found in the MorayBeyg propolis.

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