Terpenoid Compounds and Anti- Hemozoin and Anti- Ciliates Protozians Effects of *Artemisia annua* L. and *Chenopodium botrys* L.

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Abstract

Background: β-Hematin (Hemozoin) was synthised inside human erythrocyte by malaria parasite. The parasite avoids the toxic effects by polymerizing heme molecules into insoluble crystalline β-Hematin. *C. botrys* and *A. annua* used for the treatment of diseases like malaria, hepatitis, cancers, and inflammations.

Objective: Determine of antimalarial and anti-protozoa effects of *A. anuuae* and *C. botrys*.

Method: *C. botrys* and *A. annua* terpenoids were extracted by acetonitrile. The antimalarial activity of plant extracts was evaluated by in vitro β-Hematin formation. Some ciliates were tested by 0.01, 0.1 and 1 mgml^{-1} of *C. botrys* and *A. annua* extracts. Total terpenoid were measured by spectrophotometry method. The terpenoid extracts were determined by TLC and GC-MS.

Results: Terpenoids effects on *Paramecium* and other ciliates was movement inhibition and cell targeting and cilia isolating induction and cell disjointing. Anti-malarial study on β-Hematin formation showed that acetonirile and aquatic extracts of shoots and roots of two species are antimalaria. Inhibiting effects of acetonirile and aquatic extracts of *A. annua* shoots were much of double effects of shoots of acetonirile extracts of *C. botrys* and aquatic extracts of *A. annua* root. Total terpenoids of two plants were 0.317- 4.46 mgg^{-1}dw. TLC and GC/MS analysis showed that the acetonirile extract of *A. annua* contains artemisinin and several kinds of terpenoids, but in extracts of *C. botrys* with highe terpenoids content didn't find artemisinin.

Conclusion: The inhibiting effects of *C. botrys* on some ciliates and *in vitro* β-Hematin formation is from other terpenes in the extracts probably.

Keywords: β-Hematin, Hemazoin, Parasite, Terpenes

Introduction

Malaria is one of the most common infectious diseases that are caused by parasites of the genus *Plasmodium* and kills more than one million individuals in the tropical and subtropical zones annually [1, 22]. About 3.4 billion people are at risk of malaria infection in about 109 countries and territories [3]. In 2012, there were an estimated 207 million cases of malaria with over 627,000 deaths [4]. Infections are mainly caused by five species of the genus *Plasmodium*, making *P. falciparum* the most infectious parasite, contributing to 90% of total malarial deaths [2, 5]. These parasites undergo a series of morphological transformations during their life cycle. In the human host the parasites enter the liver cells, where they continue aturation before they are released into the bloodstream, where another stage called the intra-erythrocytic stage is formed. Inside of the erythrocyte the malaria parasite changes into a form called the "ring stage", at which the parasite degrades hemoglobin for its biosynthetic requirements. Large amounts of free heme known as ferriprotoporphyrin IX (FePPIX) are released [6, 22]. The accumulation of ferri protoporphyrin IX causes the generation of reactive oxygen species which may induce oxidative stress leading to parasitic death. The parasite avoids these toxic effects by polymerizing these heme molecules within the food vacuole at a pH 4.5 - 5.0, into a nontoxic, un-reactive, insoluble crystalline compound called hemozoin or "malaria pigment [2]. Hemozoin formed in this unique life cycle is considered an important target in the search of new antimalarial drugs [7, 8]. A synthetic analogue to hemozoin called β-hematin is considered to be structurally and spectroscopically identical to purified hemozoin [7] making it an excellent target for biochemistry studies. During intraerythrocytic phase of the malaria life cycle hemoglobin is utilized as a predominant source of nutrients. The amino acids derived from digestion of hemoglobin are incorporated into parasite proteins and may also be utilized for energy metabolism. Massive degradation of about 5mM hemoglobin releases large amount of toxic free heme [8]. Continuous degradation of hemoglobin and concomitant detoxification of heme are absolutely necessary for uninterrupted growth and proliferation of the parasite. Therefore, the metabolic functions related to hemoglobin digestion and hemedetoxification pathways may be potential targets for new antimalarial drug discovery [7, 8]. Protease inhibitors, which can inhibit hemoglobin digestion, have shown promising antimalarial properties *in vitro* as well as *in vivo* [7]. Recently, many strains of *P. falciparum* formed resistance to classes of pharmaceutical antimalarial drugs. *Paramecium* is one of the most abundant ciliates found in freshwater. It is covered with cilia that are used to move the *paramecium* [9]. The antimalarial drugs, artemisinin [10] and quinine, share structural similarities with compounds that inhibit ionic currents [11]. Therefore, we hypothesized that antimalarial drugs, like artemisinin, inhibit calcium-dependent backward swimming and calcium currents in *paramecium* and possess in cell membrane [12, 13]. Artemisinin is a sesquiterpene lactone and a highly selective inhibitor of a parasites Ca2+ transporting ATP-

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ases and its role is to reduce cytosolic free calcium concentrations by actively concentrating Ca2+ into membrane bound stores, an activity critical to cellular survival [10, 14, 15].

A. annua and *C. botrys* are two of the most popular herbs in traditional medicines and mostly used for the treatment of diseases like malaria, hepatitis, cancers, and inflammations [16]. There has been growing attention to *A. annua* since the isolation of artemisinin, and its distinguished clinical effects as a potent antimalarial drug [17]. *A. annua* L. is an annual medicinal and aromatic herb that belongs to the family Asteraceae [18- 20]. It is grown for its aromatic and medicinal leaves, which yields artemisinin and essential oil. Artemisinin has been proven to be potent and effective medicine for the treatment of malaria including cerebral malaria and multi drug-resistance *P. falciparum* [21- 23]. Artemisinin also acts by blocking free heme biocrystallization (like 4-aminoquinolines) and hemoglobin degradation [24].

C. botrys L. from Chenopodiaceae family is a sticky, strongly aromatic annual with an incense like odour and characteristically lobed, oaklike leaves and with taproots [25] and native plants of western Asia and Iran [26]. *C.botrys* is an annual or biennial herb and has various uses in traditional medicine [25]. It is rich in essential oil. Different isomers of ascaridole were identified in *C. botrys* oil from different origins. In some reports, these compounds were major constituents of the essential oil. Ascaridole has various properties including anthelmintic, antifungal, antimalarial, sedative and pain-relieving [27, 28].The antimicrobial and antiparasite activity of *C.botrys* oil is also recorded [29, 30]. Animalarial terponoids include artemisinin from *A. anuuae* [17, 28] and ascaridole from *C. botrys* were the objective of this work to determine the terponoids and artemisinin content and antimalarial and anti-protozoa effects of *A. anuuae* and *C. botrys* extracts.

Materials and Methods

Chemicals

Artemisinin, hemin porcine, chloroquine diphosphate, sodium dodecyl sulfate (SDS), sodium acetate, magnesium sulfate, sodium hydrogen phosphate, sodium hydroxide, glucose, and sodium bicarbonate were purchased from Sigma-Aldrich Chemical Company, oleic acid from Fluka, dimethyl sulfoxide, hydrochloric acid, acetonitrile and silica gel 60 (0.040–0.063 mm) from Merck.

Plant Materials

Artemisia annua, collected from Lahijan of Giulan province and *Chenopodium botrys* collected from Saveh of Markazi province of Iran, in September 2016. A voucher specimen of each species (number Sav-Ar-1039 and Sav-Ch-1052) was identified and deposited by Dr. B. Delnavaz Hashemloian at the central Herbarium of Saveh University of Iran. All plants were at full flowering and fruiting stages. Aerial and roots organs of 2 species plants were used for experimentations.

Terpenoid Extraction

- Crude terpenoid extraction was performed by refluxing 10 g of dry plant powder with 250 ml of hot distilled water at 60 ◦C for 2 h [31].

- Purified terpenoid extraction was performed by refluxing 10 g of dry plant powder with 250 ml of petroleum ether at 60 ◦C

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for 2 h, following a modified procedure [32]. The petroleum ether extracts were dried by vacuumed rotary evaporator at 50°C. Dried extract was further solved in 60 ml mixs of acetonitrile (20%) and hexane (30: 30) and partitioned by divider decanter to get acetonitrile fraction, containe terpenoid. All the extracts were dried using vacuumed rotary evaporator at 50°C. After complete solvent evaporation, each of these extract was solved in 5 ml absolute ethanol (terpenoids are unsolvable in water but they are solved in water by mix with ethanol) and stored in airtight bottles for further use.

In vitro β-Hematin Formation Assay

B-Hematin has been prepared chemically in high yield by abstraction of HCl from hemin porcine with a non-coordinating base in strictly anhydrous conditions. The potential anti hemozoin activity of plant extracts was evaluated by the method described by Fitch *et al.* (1999) with some modifications by Tripathi *et al.* 2004 [33, 34]. Briefly, 0-2 mg/ml in 10% DMSO [31] of the extracts were incubated with 300 µM of hemin (freshly dissolved in 0.1 M NaOH), 10 mM oleic acid and 10 µM HCl. The reaction volume was adjusted to 1000 µl using 500 mM sodium acetate buffer, pH 5. Chloroquine diphosphate was used as a positive control. The samples were incubated overnight at 37 °C with regular shaking. After incubation, samples were centrifuged (14,000 x g, 10min, at 21 °C) and the hemozoin pellet repeatedly washed with sonication (30 min, at 21 °C; FS100 bath sonicator; Decon Ultrasonics Ltd.) in 2.5% (W/V) SDS in phosphate buffered saline followed by a final wash in 0.1 M sodium bicarbonate, pH 9.0, to remove free hemin chloride, until the supernatant was clear (usually 3-5 washes).

After the final wash, the supernatant was removed and the pellets were resuspended in 1 mL of 0.1M NaOH before determining the hemozoin content by measuring the absorbance at 400 nm using a 1 cm quartz cuvette. The results were recorded as % inhibition (*I%*) of heme polymerization/crystallization compared to positive control (chloroquine) using the following formula: $I\% = [(AB–AA)/AB] \ 100$, where *AB*: absorbance of negative control; *AA*: absorbance of test sample.

Anti-ciliates and Anti-paramecium activity assay

Anti-ciliates: The water of Anzali lagoon from Giulan province of Iran were used for anti ciliates activity assay. Protozoa were recognized by some atlases and references [35- 37].

Some ciliates from coastal water of Anzali lagoon were tested by 1, 2 and 3 drops containing 0.01, 0.1 and 1 $mgml^{-1}$ of *Artemisia* and *Chenopodium* terpenoid (acetonitrile extract). 1, 2 and 3 drops of extracts were added to surface of microscopy slides and let to extract drying. After slide drying, anti protozoa effects were studied by adding 1 drop of water of Anzali lagoon.

Anti-paramecium: The medium preparation for paramecium: For preparation of paramecium, fresh leaves (10 g) of medicgo and trigonella was added to 20 ml water. Three paramecium species were presented in medium after a week. Waste leaves were isolated from medium and Anti-paramecium activity of extracts of *A. annua* and *C. botrys* was studied

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by medium solution residue contain paramecium. 1, 2 and 3 drops of extracts were added to surface of microscopy slides. Anti-paramecium effects were studied by adding 1 drop of paramecium medium to slide, after extract drying.

Total Terpenoids

Amount of 10 mg of artemisin standards were placed in a 10 ml flask and dissolved in 10 ml ethanol. Six additional calibrations were prepared by 1:2 serial dilutions with ethanol: acetonitrile (50: 50). Standard solutions were prepared 0 -200 µg ml^{-1}. Total Terpenoid were measured at 292 nm by spectrophotometry (UV- Visible Shimatzu) method [38].

Statistical analysis

For all assays, data were expressed as means ± S.E. and differences at $P<0.05$ were considered statistically significant using SPSS-16. Differences in means were estimated by means of repetitive measures followed by Dunnet's test and expressed as statistical mean ± standard deviation.

Thin Layer Chromatography (TLC)

200µl of extracts of plants were applied on to TLC plates. TLC solvent systems routinely used was petroleum ether: ether (2: 3) and TLC plates were 0.2 mm thick silica gel aluminum backed plates. Terpenoids compounds were identified using TLC and aniline blue reagent (10 mg aniline blue in 100 ml high concentration sulphuric acid).

GC-MS analysis of plant extract [39, 41].

0.1 µl of prepared extract (terpenoid) was injected to GC-MS (HP Agilent 6800 N/ (61530 N) with CPSil5 CB column (Chrompack, 100% dimethyl polysiloxane 60 m, 0.25 mm (ID), film thickness 0.25 micron). The analysis was performed under temperature programming from 100 °C (3 min) to 250 °C (5 min) with the rate of 3 °C/min. Injector split temperature was 230 °C. Identification of spectra was carried out by study of their fragmentation and also by comparison with standard spectra. Percentage of each compound was used for determination of composition amount (mg) in dry weight of plant (g):

$$Ca = Pc\ [De\ (\text{mg})/P\ (\text{g})]\ 100$$

Ca: Composition amount (mg); *Pc*: Percentage of each compound from GC analysis; *De*: dried extract (mg); *P*: plant (g)

Results

***In vitro* β- Hematin (Hemazoin) formation inhibitory**

The terpenoid extracts of aerial parts of *A. annua* and *C. botrys* was showed maximum β-hematin formation inhibitory. β- Hematin formation inhibitory of 1 mg of terpenoid extracts of *A. annua* was about 0.477 mg of hematin (I%=47.7) but for *C. botrys* was 0.28 mg ((I%= 28), respectively (Table 1). Difference of means between *A. annua* with *C. botrys* was significant inhibition of heme biocrystallization. B-Hematin formation inhibitory of chloroquin phosphate was similar to aquatic extract of aerial parts and terpenoid extract of roots of *A. annua* and terpenoid extracts of aerial parts of *C. botrys.* B-Hematin formation inhibitory of aquatic extract of aerial parts and terpenoid extract of roots of *C. botrys* was a little.

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Anti-ciliate and anti-paramecium activity

Some zooplanktons founding in Anzali lagon water include hard shells, insect's larva, nematodes, ciliates and flagellates protozoa. The acetonitrile (ace) extracts of *A. annua* and *C. botrys* don't effected on hard shells, insects larva and nematodes and some mobile unicellures. The inhibitory effects of terpenoid extracts on ciliates organisms were very highe and there are because of their die down.

The mobiling and life of all speceise of *paramecium* were inhibited by 1 $mgml^{-1}$ terpenoid extracts of *A. annua* and all concentrations of *C. botrys*. 20 seconds after *C. botrys* extract adding, deathful effect on *Paramecium aurelia* occurred during some steps. The steps includeed cell immobiling and targeting, cell deforming, cilia isolating and cell disjointing (Fig. 1). This processes have occurred for another ciliate unicellular for example, *Schistosoma sp. Urotricha platystoma* and *Stichotricha secunda* (Fig. 2, 3).

Table 1- B-Hematin formation Inhibitory of aquatic (aqu) and terpenoid (ter) extracts (Ext) of aerial (aer) and root(ro) organs(org) of *A. annua* and *C. botrys* and chloroquin phosphate (Co+):(1mg sample extract (Smg)= mg B-Hematin formation inhibitory (Hmg))

Ext	org	*A. annua*: I (Hmg/Smg)	*C. botrys:* I (Hmg/Smg)
ter	aer	0.477±0.062a	0.280±0.005b
ter	ro	0.196±0.004b	0.020±0.001c
aqu	aer	0.247±0.055b	0.047±0.014c
Co+	-	0.138±0.002b	0.138±0.002b

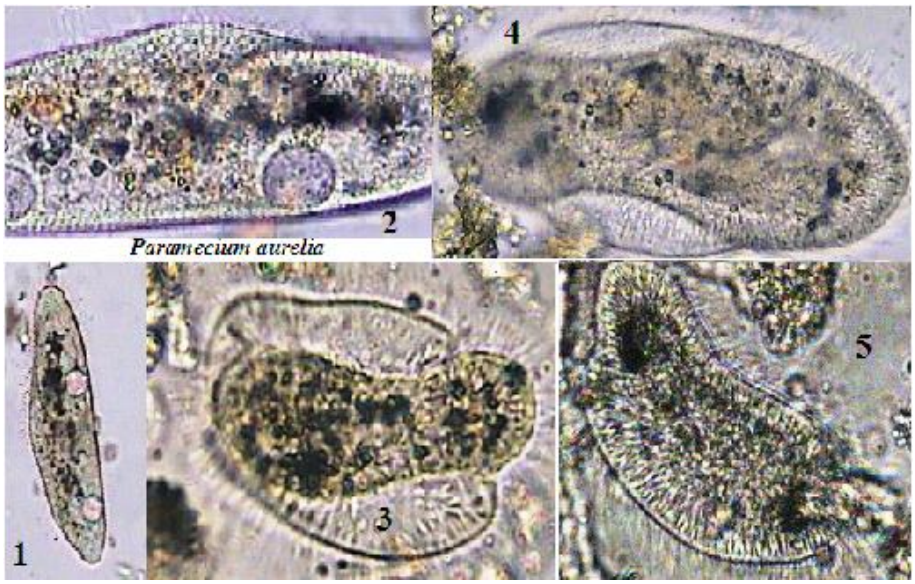

Figure 1- Deathful effect of *C. botrys* extract (1 $mgml^{-1}$) on *Paramecium Aurelia.* 20 seconds after extract adding: normal *P. aurelia* (1), cell immobiling and targeting (2), cell deforming (3, 4) and cilia isolating and cell disjointing (5).

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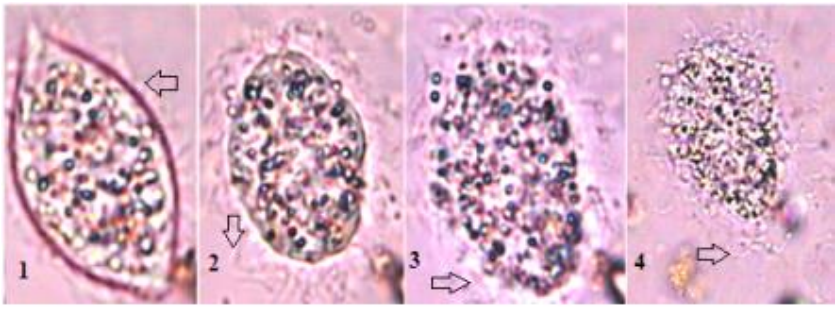

Figure 2- Deathful effect of *C. botrys* and *A. annua* extract (1 *mgml*$^{-1}$) on ciliate miracidium of *Schistosoma spp.* 20 seconds after extract adding: cell targeting (1), cilia isolating (2) and disjointing (3, 4).

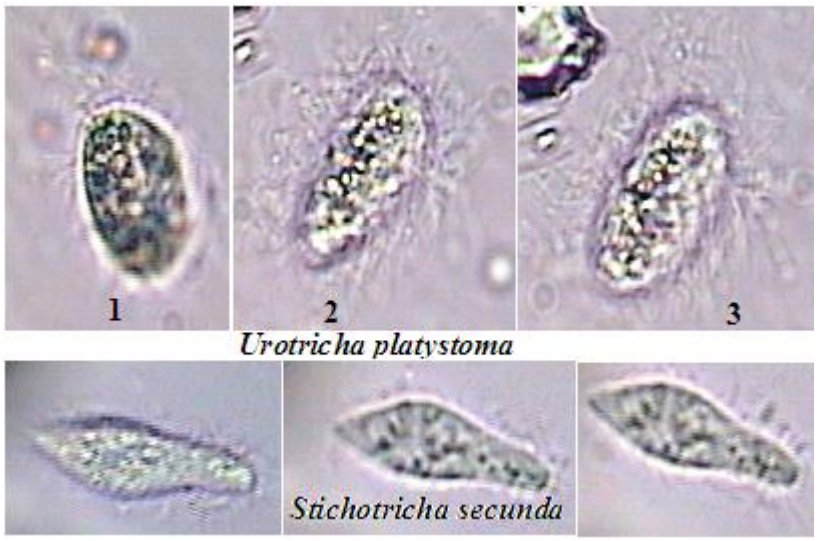

Figure 3- Deathful effect of *C. botrys* and *A. scoparia* extract (1 *mgml*$^{-1}$) on *Urotricha platystoma, Stichotricha secunda,* 20 seconds after extract: cell targeting (1), cilia isolating (2) and disjointing (3, 4).

Total terpenoid

Crude terpenoid (aquatic) was extracted from aerials parts of *A. annua* and *C. botrys* and purified terpenoid was extracted from aerials parts and roots of them with petroleum ether-hexane- acetonitrile. The terpenoid and aquatic extracts from aerials and roots of *A. annua* and *C. botrys* were yellow color. The means of total terpenoid content of purified terpenoid aerials parts of *A. annua* and *C. botrys* in dried weight (dw) of plant were 4.46 and 2.517 (mgg^{-1}dw) and in roots 0.6317and 0.317 (mgg^{-1}dw). The aquatic extracts of them were 3.70 and 1.277 (mgg^{-1}dw) respectly. The variance analysis of terpenoid contents of *A. annua*, *C. botrys* and their roots (Table. 2) showed significant difference. There was significant difference between terpenoid content of aquatic and purified extract of *A. annua* with *C. botrys*.

TLC analysis

Result of TLC showed the pattern of some terpenoids of acetonitrile extract of aerial parts *A. annua* and *C. botrys* were similar (48%

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Table 2- Total terpenoid content of of aquatic (aqu) and terpenoid (ter) extracts (Ext) of aerial (aer) and root (ro) organs (org) of *A. annua* and *C. botrys*

Ext	org	*A. annua* (mg.g.$^{-1}$)	*C. botRy* (mg.g.$^{-1}$)
ter	aer	4.46±0.025a	2.517±0.22c
aqu	aer	3.70±0.058b	1.277±0.19d
ter	rot	0.6317±0.092e	0.317±0.033f

Table 3- Results of TLC analysis: Detection of terponoids variety in acetonitrilic extraction of aerial parts *A. annua* and *C. botrys.* Terpenoid TLC analysis were showed similarity and variety between *A. annua* and *C. botrys*.

Plant	Terpenenoids Number																			
A. annua	1	2	3	4	4A	-	5A	-	7A	7	8	8A1	9	9A	10	11	12	13	-	15
C. botrys	1	2	3	4	-	5	-	6	-	7	8	-	9	-	10	11	12	13	14	15

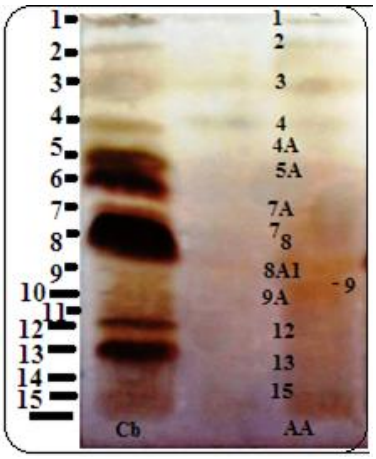

Figure 4- TLC result of acetonitrile extracts of *C. botrys* (Cb) and *A. annua* (AA): Terpenoids number of 1-4, 7-13 and 15 were common between two species. Terpenoids number of 4A, 5A, 7A and 9A presented only in acetonitrilic extraction of *A. annua.* and 14, 5 and 6 presented only in acetonitrilic extraction of *C. botrys.*

similarty). This similarity between *A. annua* and *C. botrys* was 43% (Table 3, Fig. 4). The some terpenoid include 4A, 5A, 7A, 8A1 and 9A were observed in *A. annua* and terpenoid 8A1 was artemisinin (was companied with standard artemisinin) in TLC pattern of terpenoids isolation.The results of TLC showed that artemisin absent in *C. botrys.* The terpenoid similarity of *A. annua* from Asteraceae familly with *C. botrys* from Chenopodiaceae family, was 46.7%.

GC-MS analysis: composition and quantitation terpenoids of acetonitrile extracts of aerial parts of *A. annua* and *C. botrys*.

The extraction yield for the terpenoid (acetonitrile) extraction of *A. annua* and *C. botrys* were 3.70, and 1.277 mgg^{-1}dw, respectly (Table 4). By gas chromatography mass spectroscopy (GC-MS) analysis the components of the acetonitrile extracts were identified. The acetonitrile extracts analysis led to the identification of 54 and 60 constituents for *A. annua* and *C. botrys* respectly. The GC-

MS showed different peaks of the acetonitrile extracts constituents (Table 4).

The major constituents of acetonitrile extracts of *A. annua* were Camphor (1.779 $mgg^{-1}dw$), Artemisinin (0.365 $mgg^{-1}dw$), 1,8-Cineole (0.3471 $mgg^{-1}dw$), Artemisia alcohol (0.3300 $mgg^{-1}dw$), Camphene (0.2581$mgg^{-1}dw$), Caryophyllene oxide (0.2520 $mgg^{-1}dw$), Spathulenol (0.1810 $mgg^{-1}dw$), α – Pinene (0.1155$mgg^{-1}dw$), Ledenoxid (0.1132 $mgg^{-1}dw$) and Artemisia ketone (0.099 $mgg^{-1}dw$). The major constituents of acetonitrile extracts of *C. botrys* were Elemol acetat (0.28 $mgg^{-1}dw$), α-Cadinol (0. 155 $mgg^{-1}dw$), Elemol (0.121 $mgg^{-1}dw$) and Selina-3, 11-dien-6α-ol (0.082 $mgg^{-1}dw$). Some compounds of acetonitrile extract of *C. botrys* were main substrate of artemisinine biosynthesis but there aren't any artemisinin. Artemisinin was found only in *A. annua* extract (Table 4). The acetonitrile extract compounds similarity of *A. annua* and *C. botrys* was 4.3%.

Table 4- The chemical composition of the acetonitrile extracts of *A. annua* and *C. botrys* (mg in g plant dry weight (mgg^{-1})

	C. botrys			***A. annua***		
N.	Components	RI	(mgg^{-1})	Components	RI	(mgg^{-1})
1	α-Phellandrene	1002	0.055	α – Thujene	919	0.0103
2	δ -3-carene	1007	0.0065	α – Pinene[1]	926	0.1155
3	α-Terpinene	1014	0.002	Camphene	941	0.2581
4	*o*-cymonene	1022	0.0013	Sabinene	967	0.0056
5	Limonene	1024	0.004	Myrcene	970	0.0701
6	β-*Z*-ocimene	1032	0.0049	4-Carene	972	0.0027
7	γ-Terpinene	1054	0.001	β -Pinene	974	0.0053
8	*cis*-Sabinene hydrate	1065	0.004	Dehydro–1,8-cineole	981	0.0031
9	Fenchone	1083	0.004	δ -3- carene	997	0.0242
10	β-Pinene oxide	1154	0.0065	α - Terpinene	1008	0.0078
11	Borneol	1165	0.0014	Cymol	1017	0.0483
12	Terpinene-4-ol	1174	0.004	1,8-Cineole	1024	0.3471
13	α-Terpineol	1186	0.001	γ-Terpinene	1049	0.0141
14	Bornyl acetate	1284	0.0002	Artemisia ketone	1055	0.0990
15	δ-Elemene	1335	0.0045	*cis*- Sabinene hydrate	1058	0.0107
16	α-Terpenil acetat	1346	0.0065	Terpinolene	1078	0.0470
17	α-Cubebene	1346	0.0065	Artemisia alcohol	1082	0.3300
18	α-Copaene	1374	0.001	*trans*-Sabinene hydrate	1094	0.0174
19	β-Elemene	1389	0.0369	Camphor	1148	1.7790
20	α-Gurjunene	1409	0.0009	Pinocarvone	1151	0.0784
21	*trans*-*E*-Caryophyllene	1417	0.003	Borneol	1162	0.0930
22	β-Copaene	1430	0.0005	Terpinne-4-ol	1170	0.0515
23	γ-Elemene	1434	0.003	α-Terpineol	1176	0.0450
24	α-Guaiene	1437	0.0005	Myrtenal	1184	0.0270
25	α-Hummulene	1452	0.001	Myrtenol	1187	0.0341
26	γ-Muurolene	1478	0.008	*trans*-Carveol	1209	0.0291

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Table 4- Continue

	C.botrys			*A.annua*		
27	β-Selinene	1485	0.008	*cis*-Carveol	1219	0.0153
28	10,11-epoxy-calamenene	1491	t	Carvone	1225	0.0031
29	α-Selinene	1498	0.02	Pregeijerene	1282	0.0192
30	α-Muurolene	1500	0.006	Eugenol	1342	0.0065
31	Germacrene A	1508	0.0024	α–Neoclovene	1442	0.0044
32	γ-Cadinene	1513	0.015	α -Copaene	1375	0.0122
33	δ-Cadinene	1522	0.0234	β -Bourbonene	1378	0.0051
34	*trans*-Cadina-1,4-diene	1533	0.0024	β -Cubebene	1381	0.0031
35	α-Cadinene	1537	0.0055	β -Caryophyllene	1385	0.0152
36	Selina-3,7(11)-diene	1545	0.0024	α-Hummulene	1390	0.0033
37	Elemol	1548	0.121	Farnesene	1447	0.0460
38	β-Calacroene	1564	0.0009	α-Selinene	1450	0.0530
39	Palustrol	1567	0.0022	Calarene	1455	00.005
40	α-Cederene epoxide	1574	0.034	α-Amorphene	1459	0.0030
41	Cariophyllene oxide	1582	0.0035	Germacrene D	1462	0.0110
42	Viridiflorol	1592	0.0069	β – Selinene	1468	0.0371
43	Ledol	1609	0.0107	Artemisinin	1486	0.365
44	γ-Eudesmol, 10-*epi*	1622	0.0059	Germacrene A	1507	0.0200
45	γ-Eudesmol	1630	0.022	δ-Cadinene	1518	0.0055
46	Selina-3,11-dien-6α-ol	1642	0.082	Caryophyllene oxide	1522	0.2520
47	α-Cadinol	1653	0.155	γ-Selinene	1526	0.0334
48	Selin-11-en-4α-ol	1658	t	Vulgarol B	1532	0.0331
49	Elemol acetat	1680	0.28	Aromadendrene	1538	0.0230
50	Botrydiol	1689	0.015	Isospathulenol	1557	0.028
51	Eudesm-7(11)-en-4-ol	1700	0.019	Dehydroaromadendrene	1560	0.0082
52	Guaiol acetat	1725	0.008	Spathulenol	1564	0.1810
53	β-Costol	1766	0.009	Ledenoxid	1611	0.1132
54	γ-Eudesmol acetat	1783	0.0052	δ-Cubenol	1615	0.0681
55	α-Eudesmol acetat	1794	0.046			
56	β-Chenopodiol	1811	0.015			
57	α-Chenopodiol	1855	0.031			
58	β-Chenopodiol-6-acetat	1890	0.008			
59	4α-Acetoxy-eudesman-11-ol	1938	0.0065			
60	α-Chenopodiol-6-acetat	1960	0.024			

Discussion

In view of evidence suggesting that haemozoin inhibition is the likely basis of action of a number of antimalarial drugs, considerable effort has been expended in developing assays for screening compounds for their ability to inhibit haematin formation. In this study the extracts of *A. annua* and *C. botrys* were assessed for potential antimalarial activity using the heme biocrystallization and inhibition assay. The terpenod extracts of aerial parts of both plants showed significant inhibition of heme crystallization properties. Difference of means

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between *A. annua* with *C. botrys* was significant inhibition of heme crystallization. The aquatic extracts of both plants showed leser inhibition of hemozoin formation. B-Hematin formation inhibitory of chloroquin phosphate was similar to aquatic extract of aerial parts and terpenoid extract of roots of *A. annua* and terpenoid extracts of aerial parts of *C. botrys.* These results are according some reports.Malaria-infected erythrocytes are characterized by a high rate of production of ferriprotoporphyrin IX (heme) as a result of the ingestion and digestion of host cell haemoglobin. Ferriprotoporphyrin (IX) is considered to be highly reactive and toxic to plasmodium [7, 8]. Heme affects cellular metabolism by inhibiting enzymes, peroxidizing membranes and producing oxidative free radicals [2, 34]. An important mechanism for the detoxification of heme is the formation of hemozoin in the food vacuoles of malaria parasites [2]. Hemozoin formation thus is considered an important target in the search and finding of new antimalarial drugs [7, 8]. Several studies have shown that chloroquine and most of other antimalarial compounds inhibit β- hematin formation under different conditions [33, 34]. Quinine (from *Cinchona* tree bark) would accumulate inside the food vacuole of the parasite preventing the formation of hemozoin and killing the parasite [7, 8]. Artemisinin is an endoperoxide which is the active ingredient of the *Artemisia annua* plant. Artemisinin and some other terpenoids have been proven to be potent and effective medicine for the treatment of malaria including cerebral malaria and multi drug-resistance *P. falciparum* [21- 23]. They also acts by blocking free heme biocrystallization (like 4-aminoquinolines) and hemoglobin degradation [24, 25].

The resistance to pharmaceutical products derived from these natural molecules, like chloroquine and artemether, highlights the need for new drugs. Earlier attempts showed the effect of pyrimidine derivatives, cis-platin complexes and wild sage in *in vitro* inhibition of β-hematin formation [31]. Some speciese of Artemisia genus have been and are still widely used as antimalarials [44]. They all contain a broad range of essential oils (terpenoid) which differ from species to species. It was found that α-thujone and β-thujone represent the major constituent of some artemisia species and *C. botrys* [21- 23]. Another study showed that oral delivery of dried *Artemisia annua* leaves in the form of tablets or capsules would reduce parasitaemia more effectively than a dose of pure artemisinin drug [25]. Normal isolation of essential oil performed through Hydro-destillation and by Clevenger apparatus but in this study performed by disstiled water and petroleum ether- acetonirile. The acetonitrile extracts containing full terpenoids had stronger activity and showed broader spectrum results against some ciliate organisms. The effects of terpenoids extracts of *A. annua* and *C. botrys* on paramecium and other testing ciliates were inhibiting of movement, cell targeting, cilia isolating and cell disjointing. These results are according some reports. Plants of the genus *Artemisia*, were described as having antiprotozoal, antibacterial and antifungal effects [42-47]. *A. annua* is grown on an industrial scale in many parts of the world for the manufacture of therapeutic combinations based on artemisinin used in the treatment of

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malaria [19]. Artemisinin of *A. annua* has been identified as the anti-malarial principle of the plant, and artemisinin derivatives are nowadays established as anti-malarial drugs with activity towards otherwise drug-resistant *Plasmodium* infections. Artemisinin, a bioactive molecules of the plant proved effective against *schistosomes*, flukes 60 and some viruses [48]. This molecule inhibits the development of *Cryptosporidium, Giardia intestinalis, Entamoeba histolytica*, and various species of *Leishmania*. Macsimovic *et al*. (2005) reported that the essential oil isolated from aerial parts of collected *C. botrys* exhibited parasiticidal, bactericidal and fungicidal activity [49].

The phyla of ciliates (*Paramecium* and *Tetrahymena*) and Apicomplexa (*Plasmodium* and *Toxoplasma*), together with dinoflagellates, are united in the superphylum Alveolata [50]. *P. falciparum*is a protozoan parasite which can infect humans, causing the disease malaria [51]. Toxic effects of antimalarial drug on ciliate may be mediated through inhibition of calcium currents. *Paramecia* were viable in low concentration of quinacrine, a drug with hiegher concentration that substantially stoped backward swimming and the calcium current [52]. *P. falciparum,* into the red blood cell is inhibited by antimalarial drugs. If antimalarials inhibit calcium currents in *Plasmodium,* the drugs may be effective in blocking invasion of the parasites into the red blood cell and survival of the parasites once within them. The some antimalarial drugs inhibit potassium currents in protozoan and metazoan cells [53]. The antimalarial drugs also inhibits calcium-dependent backward swimming and calcium currents in *Paramecium.* The difference in potency of the drugs in inhibiting backward swimming and calcium currents may indicate that the drugs reduce backward swimming in *P. calkinsi* in part by inhibition of calcium currents and in part by other mechanisms [54]. The antimalarial share structural similarities with compounds that inhibit ionic currents. The antimalarial drugs, like W-7, inhibit calcium-dependent backward swimming and calcium currents in paramecium [11].

The chemical studies on Artemisia species indicate that all classes of compounds are present in the genus with particular reference to terpenoids and flavonoids. The rich accumulation of essential oils and other terpenoids in the Artemisia genus is responsible for the use of various members for medicinal, flavouring foods or liqueurs [26-42]. During the recent decade's terpenoids and sesquiterpenoid lactones have emerged as one of the largest groups of plant products with over 3000 naturally occurring substances known. The some species of Artemisia from different parts of Iran were tested for terpenoids and sesquiterpenoid lactones. The findings revealed that all species of *Artemisia* had a high amount of terpenoids and sesquiterpenoid lactones [55]. Terpenoid content of aerial portion of *Artemisia* genus is 2.08 $mgg^{-1}dw$ [56]. In this study Terpenoid content of acenitrile extracts of aerials parts of *A. annua* and *C. botrys* were 4.46 and 2.517 ($mgg^{-1}dw$) and aquatic extracts of them were 3.70 and 1.277 ($mgg^{-1}dw$) and in their roots 0.6317 and 0.317 ($mgg^{-1}dw$) respectly. Artemisinin content of terpenoid extract of *A. annua* was 0.365 $mgg^{-1}dw$. Artemisinin weren't find in terpenoid extract of *C. botrys* by GC/MS analysis. Artemisinin was found only

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in *A. annua* terpenoid extract. Some compounds of acetonitrile extract of *C. botrys* were main substrate of artemisinine biosynthesis but there weren't any artemisinin. *A. annua* is the only commercial source of artemisinin. At the moment, however, lower content (0.01–0.8%, dw) of artemisinin in leaves and flowers of *A. annua* has seriously limited its commercialization and triggered numerous efforts for improving artemisinin production [57]. Artemisinin content of *A. annua* has reported in the range of 0.090 – 0.330 mgg^{-1}dw) in the aerial portion of the plant [58]. But in others is 0.52- 0.91 mgg^{-1}dw [59]. In this study, artemisinin content of aerial portion of *A. annua* was 0.365 mgg^{-1}dw.

Result of TLC showed the pattern of some terpenoids bands of acetonitrilic extract of aerial parts *A. annua* and *C. botrys* are similar (46.7% similarty). Many secondary metabolites of terpenes such as artemisia ketone, artemisinic alcohol, arteannuin B and myrcene hydroperoxide, over 50 different phenolic compounds belonging to five major groups (flavones, flavonols, coumarins, phenolic acids, and a miscellaneous group) which the main coumarins are coumarin, aesculetin, iso-fraxidin, scopoletin, scopolin, tomentin, and also other phenolic compounds such as 2, 4-dihydroxy-6- methoxy-acetophenone, 5-nonadecyl-3-O-methyletherresorcinol, 2, 2, 6-trihydroxychromene and 2, 2-dihydroxy-6-methoxychromene have been isolated of this plant [60]. Artemisinin (sesquiterpene lactone) is a quite expensive compound with very low concentration which the only commercial source available is *A. annua*. From approximately 400 species of artemisias, *A. annua* is the main source of artemisinin [61, 62].

In this study, the extraction yield for the acetonitrile of *A. annua* and *C. botrys* were 4.46 and 2.517 mgg^{-1}dw, respectly. By gas chromatography mass spectroscopy (GC-MS) analysis the components of the acetonitrile extracts were identified. The acetonitrile extracts analysis led to the identification of 54 and 60 constituents for *A. annua* and *C. botrys* respectly.

The oil of *A. annua* was rich in monoterpenoids with 83.72% monoterpenoids and 12.59 % sesquiterpenoids. The hydrodistillaton of the dried flowering aerial parts of *A. annua* L. gave a light yellowish oil with yield of 1.2 % (v/w). 32 components were identified in the oil, representing 96.83 % of the total composition. The major components of the essential oil were campher, 1, 8-cineole, camphene and spathulenol [62, 63]. The components of the essential oil extracted by hydro distillation from the stems and spikes of *A. annua* were identified using chromatography–mass spectrometry (GC/MS). The stem oil was composed of 50 components, while the spike oil contained 41 components. 30 compounds were found in both oils. In this study the major constituents of acetonitrile extracts of *A. annua* were camphor, artemisinin, 1, 8-cineole, artemisia alcohol, camphene, caryophyllene oxide, spathulenol, α-pinene, ledenoxid and artemisia ketone.

C. botrys have monoterpenes, comprise: camphor, delta-3-carene, fenchone, linalool mentone, nerol, beta- pinene, pulegone, thujone, terpineo l-4 and sesquiterpenes, comprise: beta elemene, elemol, beta eudesmol [64]. *C. botrys* is rich in essential oil and the antimicrobial activity of the oil is also recorded

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[29, 30]. Chemical composition of essential oil isolated from aerial parts of *C. botrys* L. (Chenopodiaceae) collected from five different locations in the Republic of Macedonia was analysed by GC/FID/MS. 75 compounds were identified representing 90.02-91.24% of the oil. The analysis has shown that the oils were rich in sesquiterpene components comprising elemol ace tat, seline-11-en-4α-ol, selina-3, 11-dien-6α-ol and elemol as major oxygen containing sesquiterpenes, followed by lower content of α-eudesmol acetat, α-chenopodiol, botrydiol and α-chenopodiol-6-acetat [65]. The oil of aerial parts of *C. botrys* from Isfahan, Fars, Mazandaran provinces of Iran were analyzed by using GC/MS. The 33, 19, 21 compounds were identified in dried aerial parts, respectively. 1, 8- Cineole was common major component in Isfahan, Fars and Mazandaran plants. Camphene and β-Mycene from Isfahan plants, Camphor and α-pinene from Fars plants and β- Myrcene and Camphor from Mazandaran determinate that were other major components [66]. The monoterpenoid ascaridole and other terpenoids reported from *Chenopodium* genus from the East Mediterranean [28]. In present study, the major constituents of acetonitrile extracts of *C. botrys* were elemol acetat, α-cadinol, elemol and selina-3, 11-dien-6α-ol and there was very different from other reports.

Conclusion

A. annua and *C. botrys* showed anti hemozoin formation or antimalarial effects. However, effect of *A. annua was* greater than that of *C. botrys.* Among the extracts of *A. annua* the acetonitrile extract (full terpenoids) displayed the most potent activity. *A. annua and C. botrys* have anti ciliate compouns. The GC-MS analysis has shown that the acetonitrile extracts of both plants were rich in very kind tepenoids and sesquiterpene components. Some compounds of acetonitrile extract of *C. botrys* were main substrate of artemisinine biosynthesis but there weren't any artemisinin. Artemisinin was found only in *A. annua* extract. Another compound of *C. botrys* extracts are anti-malaria and anti-ciliate effects of this plant.

Abbreviations:

TLC (thin-layer chromatography), GC-MS (Gas Chromatography- Mass) Declarations

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Availability of data and materials

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Authors' contributions

Azra conceived and designed the experiments; Azra and Babak performed the experiments; they wrote the paper and edited the study. All authors have read and approved the final version of manuscript.

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

The authors declare that they consent for publication.

Ethics approval and consent to participate

All procedures were performed by *in vitro* experiments without any human and animal usage, in laboratories of plant biology and biotechnology of Islamic Azad University of Saveh.

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