Iranian Journal of Basic Medical Sciences

ijbms.mums.ac.ir

IJBMS

Antimalarial and cytotoxic activities of roots and fruits fractions of *Astrodaucus persicus* extract

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Jul 23, 2017
Accepted: Sep 28, 2017

Keywords:
Astrodaucus persicus
Apiaceae
Antimalaria
Cytotoxic
MTT assay
Plasmodium berghei

ABSTRACT

Objective(s): *Astrodaucus persicus* (Apiaceae) is one of the two species of this genus which grows in different parts of Iran. Roots of this plant were rich in benzodioxoles and used as food additive or salad in Iran and near countries. The aim of present study was evaluation of antimalarial and cytotoxic effects of different fractions of *A. persicus* fruits and roots extracts.

Materials and Methods: Ripe fruits and roots of A. *persicus* were extracted and fractionated by hexane, chloroform, ethyl acetate and methanol, separately. Antimalarial activities of fractions were performed based on *Plasmodium berghei* suppressive test in mice model and percentage of parasitemia and suppression were determined for each sample. Cytotoxicity of fruits and roots fractions were investigated against human breast adenocarcinoma (MCF-7), colorectal carcinoma (SW480) and normal (L929) cell lines by MTT assay and IC_{50} of them were measured.

Results: Hexane fraction of roots extract (RHE) and ethyl acetate fraction of fruits extract (FEA) of *A. persicus* demonstrated highest parasite inhibition (73.3 and 72.3%, respectively at 500 mg/kg/day) which were significantly different from negative control group ($P<0.05$). In addition, RHE showed potent anticancer activities against MCF-7 (IC_{50} of 0.01 µg/ml), SW480 (IC_{50} of 0.36 µg/ml) and L929 (IC_{50} of 0.70 µg/ml) cell lines.

Conclusion: According to the results, RHE and FEA fractions of *A. persicus* could be introduced as excellent choice for antimalarial drug discovery. In addition, cytotoxic activity of RHE was noticeable.

► *Please cite this article as:*
Goodarzi S, Nateghpour M, Asgharian P, Hadjiakhoondi A, Yassa N, Tavakoli S, Mirzaei J, Farivar L, Motevalli Haghi A, Tofighi Z. Antimalarial and cytotoxic activities of roots and fruits fractions of *Astrodaucus persicus* extract. Iran J Basic Med Sci 2017; 20:1318-1323. doi: 10.22038/IJBMS.2017.9554

Introduction

Malaria is one of the most important infectious diseases globally and the major health challenges in malaria risk areas. According to WHO malaria report, there were an estimated 212 million new cases of malaria and 429000 deaths in 2015 alone worldwide. The heaviest malaria burden belongs to African region countries. Iran showed more than 40% decrease in malaria incidence and mortality rates between 2010-2015 (1).

The greatest challenges against malaria control is resistance of mosquitoes to insecticides and parasites to antimalarial drugs. From four species of malaria parasites which naturally infect humans, *Plasmodium falciparum* has developed resistance to nearly all mainstay antimalarial drugs and *P. vivax* showed resistance to chloroquine derivatives (2-4).

Considerable costs of drugs and logistical problems especially in poor malaria endemic areas were other challenges for control of disease (5).

Over the previous 30 years, natural products are the sources of approximately two thirds of all drugs introduced (6). Medicinal plants are potential sources of novel, effective and affordable antiplasmodial compounds which can elucidate chemically or can constitute lead molecules for discovery of new antimalarial drugs (7, 8). Quinine present in *Cinchona* species, artemisinin from *Artemisia annua*, quassinoids in Simaroubaceae and limonoids in Meliaceae families were natural products with considerable antimalarial activities (9, 10). The *Astrodaucus* genus from Apiaceae family is native to Iran, Iraq, Syria, Turkey and Ukraine (11).

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Two species of *A. persicus* (Boiss.) Drude and *A. orientalis* (L.) Drude distributed in various parts of Iran.

Aerial parts and roots of *Astrodaucus* used as food additive or salad in some regions of Iran and Turkey (12). New compounds with 1, 3-benzodioxole structures were isolated and elucidated from different fractions of *A. persicus* roots extract (13). Benzodioxoles demonstrated antimalarial, antioxidant, antitumor, antibacterial, antifungal, antiparasitic, pesticides and herbicides properties (14).

There was no investigation on antimalarial activities of *A. persicus*, therefore to fill this gap in, the present study was designed to evaluate antimalarial and cytotoxic activities of different fractions of fruits and roots extracts of *A. persicus*.

Materials and Methods

Plant materials

Ripe fruits and roots of *A. persicus* (Boiss.) Drude were collected in September 2015 from Zanjan Province, Iran. Plant was deposited in Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences (6553-TEH; identified by Dr Y Ajani). The samples were dried in shade and powdered separately.

Extraction and fractionation

The *A. persicus* fruits (150 g) and roots (1190 g) were extracted separately with 80% methanol by maceration at 25±2 °C to obtain crude fruits extract (FE 17 g) and roots extract (RE 42.5 g). FE and RE were fractionated with hexane (FHE 2.88 g, RHE 15.63 g), chloroform (FCL 0.76 g, RCL 7.44 g), ethyl acetate (FEA 0.33 g, REA 2.54 g) and the residue named methanol fraction (FME 11.53 g, RME 15.25 g).

Animals

The Swiss albino male mice (20-25 g) were obtained from the Pasteur Institute of Iran. The animals were housed in comfortable cages at room temperature under 12 hr light-dark cycles with free access to standard pellet diet and clean water *ad libitum*. The study performed according to the Helsinki rules in animal house of School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. All procedures performed in studies involving animals were in accordance with the Ethical Standards of Tehran University of Medical Sciences (IR.TUMS.REC. 1394.935).

Parasites

Chloroquine-sensitive *Plasmodium berghei* (NICD strain) was obtained from Haffkine Institue, India for induction of malaria in experimental mice.

Two weeks previous to the tests, mice were infected with 0.2 ml suspension of *P. berghei* via intra-peritoneal (IP) route and were used as donor.

Inoculum preparation

The parasitemia of the *P. berghei* infected donor mice were measured every day. Four days after infection, parasitemia reached about 10%. The heart blood of infected donor mice was collected via cardiac puncture into heparinized test tube after anaesthetizing animals with ketamine/xylazine.

The blood was diluted with physiological saline. The dilution was done based on the parasitemia percentage of donor mice and RBC count of normal mice (15). Mice were then infected by injecting 0.2 ml of diluted blood suspension intraperitoneally (IP) which contained 10^6 parasitized erythrocytes on day 0 (D0).

Antimalarial test

Plasmodium berghei suppressive test, a preclinical test for measurement of potential bioactivity of new compounds and extracts, was conducted for determination of the drug doses for producing 50% suppression of parasitemia (16). Mice were grouped into eleven groups of five individual. Treatment was started two hrs after injection of parasites and then continued once daily for 21 days. Group I to IV and V to VIII were treated with 500 mg/kg/day (IP) of hexane, chloroform, ethyl acetate and methanol fractions of fruits and roots extracts, respectively. Positive and negative control groups received chloroquine (10 mg/kg/day) and normal saline as vehicle (0.2 ml/kg/day), respectively. Group XI (blank) were infected mice which didn't receive anything. On the 4th day (D_4), 7th day (D_7), 14th day (D_{14}), 21th day (D_{21}), thin blood smears were prepared from the tail of each mouse on microscopic slides, then they were fixed with methanol and stained with 10% Giemsa in distilled water. The numbers of parasitized erythrocytes were counted and percentage of parasitemia and inhibition were determined for each sample. Parasitemia percent and suppression percent were calculated by following formula, respectively (17, 18).

%Parasitemia= Number of Infected RBCs/Total Number of RBCs ×100

%suppression= (%Parasitemia of BG - %Parasitemia of TG)/ %Parasitemia of BG) ×100

Where BG means blank group and TG means treated group. Thin smears of treated mice were investigated on days 7, 14 and 21 after infection, too. Results were expressed as mean±SD. One way analysis of variance (ANOVA) and Tukey *post hoc* were used for analyzing the data and $P< 0.05$ was considered significant.

Cytotoxicity evaluation

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazo - lium bromide (MTT; Sigma-Aldrich, USA); RPMI 1640, fetal bovine serum (FBS), penicillinstreptomycin (GIBCO™ Invitrogen, USA) and trypsin-EDTA (Boehringer, Germany) were prepared.

Table 1. Antimalarial effect of different fractions of *Astrodaucus persicus* roots and fruits extracts against *Plasmodium berghei* infection in mice

Treatment	Doses	%Parasitemia (%Suppression)			
		4th Day	7th Day	14th Day	21th Day
Normal saline	0.2 ml/kg	7.70±4.13 (13.3)	19.00±6.63 (26.7)	25.60±4.72 (16.3)[1]	50.50±1.32 (0.0)[3]
RHE	500 mg/kg	1.60±1.14 (73.3)*	6.80±1.56 (64.2)	26.00±1.69 (15.4)	46.75±2.47 (9.2)[3]
RCL	500 mg/kg	3.70±2.49 (38.3)	8.10±1.71 (57.4)	29.87±2.21 (2.8)[1]	48.00±2.12 (6.8)[3]
REA	500 mg/kg	4.10±1.78 (31.7)	10.10±3.58 (46.8)	29.83±3.21 (3.0)	----[5]
RME	500 mg/kg	4.25±2.96 (29.2)	11.50±8.06 (31.2)	20.37±13.62 (33.7) [1]	48.00±3.94 (8.4) [3]
FHE	500 mg/kg	4.08±0.86 (35.3)	17.66±4.40 (39.1)	19.80±4.45 (35.2) [1]	47.33±1.53 (6.3) [3]
FCL	500 mg/kg	4.70±1.44 (25.5)	18.64±2.36 (35.7)	23.00±1.41 (24.8)	37.00±0.0 (26.7)[2]
FEA	500 mg/kg	1.75±1.71 (72.3)*	14.25±9.63 (50.9)	14.75±9.84 (51.7) [3]	39.33±9.52 (22.1) [4]
FME	500 mg/kg	3.13±0.68 (50.3)	20.57±4.72 (29.1)	24.08±5.37 (21.2)	----[5]
Chloroquine	10 mg/kg	0.00±0.00 (100)	0.00±0.00 (100)	0.00±0.00 (100)	0.00±0.00 (100)

Values are expressed as mean±SD, RHE: Root hexane fraction, RCL: root chloroform fraction, REA: Root ethyl acetate fraction, RME: Root methanol fraction, FHE: Fruit hexane fraction, FCL: Fruit chloroform fraction, FEA: Fruit ethyl acetate fraction, FME: Fruit methanol fraction. *: There was significant difference with normal saline (P<0.05). The superscript number means the number of died from five mice in each group.

Table 2. Cytotoxicity of different fractions of *Astrodaucus persicus* roots and fruits extracts by MTT assay

Samples	IC_{50} (SI)		
	MCF7	SW480	L929
RHE	0.01±0.01(70)	0.36±0.1(1.9)	0.70±0.12
RCL	42.21±0.48(2.4)	71.44±0.17(1.4)	100.00±0.93
REA	250.27±0.15(>4.0)	397.93±0.12(>2.5)	>1000
RME	>1000	>1000	>1000
FHE	>1000	>1000	>1000
FCL	>1000	5.42±1.24(>184.5)	>1000
FEA	>1000	>1000	>1000
FME	>1000	>1000	>1000
Doxorubicin	0.35±0.07(1.6)	2.50±0.80(2.4)	0.55±0.06(0.2)

Results are expressed as IC_{50} value (µg/ml), mean±SD of three determinations; SI: selectivity index, MCF-7: breast adenocarcinoma, SW480: colorectal carcinoma and L929: normal cell lines., RHE: Root hexane fraction, RCL: Root chloroform fraction, REA: Root ethyl acetate fraction, RME: Root methanol fraction, FHE: Fruit hexane fraction, FCL: Fruit chloroform fraction, FEA: Fruit ethyl acetate fraction, FME: Fruit methanol fraction

Cell culture

The human breast adenocarcinoma (MCF-7), colorectal carcinoma (SW480) and normal (L929) cell lines were cultured in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C.

MTT assay

Cell viability was determined by the microculture tetrazolium/formazan assay using MTT (19). MTT (5 mg/ml) was dissolved in PBS. The solution was filtered through a 0.2 µm filter and stored at 2-8 °C. Cells were cultured in 96-well plates at density of 10^4 cells/ well in 100 µl medium at 37 °C for 24 hr. Following incubation, the medium of each well was replaced by 100 µl fresh medium containing various concentrations of different fractions of roots and fruits of *A. persicus* extract, which were freshly prepared in DMSO. The final concentration of DMSO was adjusted lower than 1% of total volume. After 48 hr incubation, the medium removed and 20 µl of MTT reagent was added to cells. The plates were incubated at 37 °C for 4 hr in a humidified 5% CO_2 atmosphere. Then the MTT was removed and pure DMSO (100 µl/well) was added to lyse the cells and dissolve formazane crystals. The purple formazane salts formed from enzymatic reduction of yellowish MTT in mitochondria of viable cells. The absorbance was measured immediately using a micro plate reader (Anthos, Austria) at 570 nm. The cell survival was calculated by the following formula:

% Cell survival = Mean absorbance in test wells/Mean absorbance in control wells × 100

The results were generated from three independent experiments; each experiment was performed in triplicate. The IC_{50} (the median growth inhibitory concentration) was calculated from a dose response curve plotted in the Sigma Plot 10 software.

The selectivity index (SI) of fractions was measured according to the following equation:

SI= IC_{50} of none cancer cells/ IC_{50} of cancer cells (20).

Results

Antimalarial activity

Chemo suppressive activity of different fractions of the roots and fruits of *A. persicus* extract against *P. berghei* infection in mice demonstrated in Table 1.

RHE and FEA were the most active fractions exhibiting 73.3 and 72.3 % suppression of *P. berghei* parasitemia on the fourth day, respectively. RHE, RCL and FEA were fractions with highest parasite inhibition (64.2, 57.4 and 50.9, respectively) on the seventh day. Statistical analysis demonstrated only RHE and FEA were significantly different from negative control group on the fourth day (P=0.045 and 0.023, respectively). RHE, RCL and FEA didn't show any significant difference from control on the seventh day (P=0.19, 0.43 and 0.78, respectively).

Cytotoxicity assay

Cytotoxicity of different fractions of fruits and roots of *A. persicus* extract were evaluated on the human breast adenocarcinoma (MCF-7), colorectal carcinoma (SW480) and normal (L929) cell lines by MTT assay and IC_{50} were demonstrated in Table 2. RHE and RCL fractions exhibited potent cytotoxic effects on MCF-7, SW480 and L929 cell lines, while REA fraction showed cytotoxicity on MCF-7 and SW480 cell lines in high doses (IC_{50} equal to 250.27 and 397.93 µg/ml, respectively). RME fraction showed no cytotoxic effects on different cell lines. From different fractions of fruits extract, only FCL showed potent cytotoxicity (IC_{50} up to 5.42 µg/ml) on SW480 cell line and other fractions didn't show any toxicity.

Discussion

Medicinal plants contain a great variety of metabolites with different structures and biological activities therefore they can be good choices for drug discovery including antimalarial drugs. Two antimalarial drugs of quinine and artemisinin, currently in use have been elucidated from *Cinchona* species and *Artemisia annua* traditionally used for malaria treatment.

In present study, antimalarial activity of different fractions of fruits and roots extracts of *A. persicus* against *P. berghei* infection were investigated in mice and cytotoxicity of them against cancer and normal cell lines were reported.

The parasitemia suppression percentage of almost all roots and fruits fractions of *A. persicus* extract were upper than 30% on fourth and seventh days. The previous investigations demonstrated when parasitemia suppression percentage were more than 30%, the compound or extract is considered as active (21, 22). Another researchers categorized *in vivo* antimalarial activity of plants extracts as moderate, good or very good when the extracts at 500, 250 and 100 mg/kg/day showed 50% or more chemosuppression, respectively (23). According to this classification, only FEA and RHE fractions with parasitemia inhibition extra 50% at 500 mg/kg were effective against malaria.

Intercalation in DNA, inhibition of protein synthesis, prevention of parasite invasion to new RBCs (24, 25), reducing parasite nutrient intake (26), antioxidant and free radical scavenging activity (17) and immunomodulatory effects (27) were proposed as mechanisms for action of antiplasmodial drugs. According to previous investigation, roots extract of *A. persicus* demonstrated potent antioxidant effects and high amounts of total phenolic compounds (12). Also there were reports about dose and time dependent antiproliferative and cytotoxic effects of both roots and aerial parts of *A. persicus* extracts (28, 29). In conclusion, both antioxidant activity and cytotoxicity could be considered as antiplasmodial mechanisms of roots extract of *A. persicus*.

Many antimalarial molecules which discovered have high toxicity and low therapeutic indices (30), Therefore cytotoxicity of different fractions of fruits and roots of *A. persicus* were evaluated.

Following the standard of Plant Screening Program of US National Cancer Institute (NCI), possessing an IC_{50} less than 20 µg/ml for crude extracts and less than 4 µg/ml for pure compounds are considered active against the tested cancer cells (19). Based on these criteria, only RHE fraction of *A. persicus* extract demonstrated potent anticancer activities against MCF-7 (IC_{50} of 0.01 µg/ml), SW480 (IC50 of 0.36 µg/ml) and L929 (IC_{50} of 0.70 µg/ml). This fraction showed potent activity against breast and colorectal cancer cell lines in comparison to doxorubicin with IC_{50} of 0.35 and 2.50 µg/ml, respectively. Cytotoxic activity of RHE on normal cell lines was almost equal to doxorubicin (IC_{50} of 0.55 µg/ml). Another investigation showed IC_{50} of cisplatin against MCF-7 was equal to 19.28 µg/ml and against SW480 was equal to 25.69 µg/ml (31). It was obvious that RHE fraction of *A. persicus* was more potent than doxorubicin as an anticancer drug. On the other side, the selectivity of RHE fraction for breast adenocarcinoma was 70 times more than normal cell line and for colorectal carcinoma was almost 2 times more than normal cell line. The previous studies demonstrated compounds with selectivity index higher than 10 was considered as selective while those with SIs lower than 10 but higher than 1 were belong to non-selective ones (20). The results demonstrated RHE could be an excellent choice for anticancer drug discovery especially for its selective effects on breast cancer. FCL fraction showed potent selectivity against colorectal carcinoma cell lines but IC_{50} of this fraction was lower than doxorubicin. It could be considered for elucidation of its compounds as leads for anticancer drugs.

Bioactive secondary metabolites containing benzodioxole structures were responsible for many pharmacological activities of plants. Lycorine, a natural alkaloid containing benzodioxole structure, isolated from Amaryllidaceae genera, was active against

chloroquine-sensitive strains of *P. falciparum* (32). Other bonzodioxole compounds like safrole, apiol and myristicin showed carcinogenic and other toxicological effects (33-35). Clinical antitumor agents like etoposide, teniposide and podophylotoxin have methylenedioxy unit in their structures (36, 37). Previous studies showed excellent bioavailability and low cytotoxicity of a variety of anticancer drugs with benzodioxole structures (38). Low cytotoxicity of molecules with benzodioxole structures converted them to considerable antimalarial compounds. Elucidation of active antiplasmodial compounds from effective fractions (RHE and FEA) of *A. persicus* extract and investigation of their toxicity were proposed for further studies.

Conclusion

The results of this study have shown potent antimalarial and cytotoxic effects of hexane fraction of *A. persicus* roots extract. On the other hand, ethyl acetate fraction of fruits showed antimalarial activity with no cytotoxicity. The existence of bioactive compound(s) in RHE and FEA fractions or the synergist effects of compounds may be the reason of such good results.

Acknowledgment

This research was a Pharm D thesis which was supported by a grant of Tehran University of Medical Sciences and Health Services (No 29070).

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