RESEARCH ARTICLE

Assessment of *Euphorbia retusa* and *Pulicaria undulata* activity against *Leishmania major* and *Toxoplasma gondii*

Khan, T.A.[1], Al Nasr, I.S.[2,3], Mujawah, A.H.[4], Koko, W.S.[3*]

[1]Department of Biomedical Instrumentation, College of Applied Health Sciences, Qassim University, Ar Rass, Saudi Arabia
[2]Department of Biology, College of Science and Arts, Qassim University, Unaizah, Saudi Arabia
[3]Department of Science Laboratories, College of Science and Arts, Qassim University, Ar Rass, Saudi Arabia
[4]Department of Chemistry, College of Science and Arts, Qassim University, Ar Rass, Saudi Arabia
*Corresponding author: wasyko2002@yahoo.com

ABSTRACT

Leishmaniasis and toxoplasmosis are parasitic protozoal diseases that pose serious health concerns, especially for immunocompromised people. *Leishmania major* and *Toxoplasma gondii* are endemic in Saudi Arabia and are particularly common in the Qassim Region. The present work was conducted to evaluate the *in vitro* antileishmanial and antitoxoplasmal activity of methanolic extracts and phytochemical fractions from two plants, *Euphorbia retusa* and *Pulicaria undulata,* which are ethnobotanical agents used to treat parasitic infection. Whole *E. retusa* and *P. undulata* plants were extracted with methanol and fractionated using petroleum ether, chloroform, ethyl acetate, n-butanol, and water and then were tested *in vitro* against *L. major* promastigote and the amastigote stages of *T. gondii*; the cytotoxicity of the extracts was tested against Vero cell line. The methanolic extracts of *E. retusa* and *P. undulata* exhibited promising antitoxoplasmal activity against *T. gondii* with EC_{50} values 5.6 and 12.7 µg mL^{-1}, respectively. The chloroform fraction of *P. undulata* was the most potent, exhibiting an EC_{50} of 1.4 µg mL^{-1} and SI value of 12.1. It was also the most active fraction against both *L. major* promastigotes and amastigotes, exhibiting an EC_{50} of 3.9 and 3.8 µg mL^{-1} and SI values 4.4 and 4.5, respectively. The chloroform fraction from *P. undulata* is a very good candidate for the isolation of active antitoxoplasmal and antileishmanial ingredients; therefore, further phytochemical analysis for active compound isolation is highly recommended.

Keywords: *Toxoplasma gondii, Leishmania major*, *In vitro, Euphorbia retusa, Pulicaria undulata*.

INTRODUCTION

Both toxoplasmosis and leishmaniasis are major parasitic diseases of global importance. They are also highly endemic in Saudi Arabia, while their prevalence is characterized by regional variation (Alsammani, 2016; Rasheed *et al.*, 2019). Leishmaniasis diseases show different types of clinical manifestations that etiologically occur from species of the genus *Leishmania*, which is contained in more than 20 species, and all of them are transmitted by infected female phlebotomine sandflies. There are three main types of leishmaniasis; visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (ML), and VL and CL are the most common types recorded and are endemic from 56 and 59 countries, respectively, as of 2020 and based on the WHO Global Leishmaniasis program for 2018 (WHO, 2020).

CL is the common form in Saudi Arabia and reported in many parts of the country because of the distribution of vectors and presence of reservoir animals (Haouas *et al.*,

2017; Al Nasr *et al.*, 2019a). This disease has been an epidemic in Al Qassim Province many times during the last two decades (Rasheed *et al.*, 2019).

In Saudi Arabia, CL is commonly caused by *L. major* species that become increasingly resistant to commonly used therapeutics (Croft & Coombs, 2003), which also have many side effects (Sundar *et al.*, 2000). For this reason, medicinal plants that also have been traditionally used without any major side effects can be considered suitable candidates for further scientific investigation towards novel drug discoveries that may show better efficacy.

Toxoplasmosis is commonly caused by *Toxoplasma gondii*, which is an intracellular pathogenic globally distributed protozoon and known to be found in almost all warm-blooded animals (Peng *et al.*, 2011; Alzaheb, 2018). This parasite infects approximately one third of the world's population and is considered one of the most successful human parasites (Pappas *et al.*, 2009). In fact, the Centers for Disease Control have prioritized *T. gondii* as one of the top "Five Neglected Parasitic Infections" due to the severity of

illness, high incidence, and potential for prevention (Li *et al.*, 2014). The infection can be fatal sue to severe symptoms in immunocomp romised people, and it can also be associated with abortions during pregnancy and highly serious congenital abnormalities (Khalil *et al.*, 2018).

Toxoplasmosis has been reported as an epidemic in Saudi Arabia several times, especially in the southwest and central parts of the country (Phillipson & Wright,1991; Al Nasr *et al.*, 2016).

Currently, there are few effective antitoxoplasmal drugs, but they have adverse side effects and require long courses of administration (Holzgrabe & Bechthold, 1999; Camacho *et al.*, 2000). However, the previous investigation on natural products, mainly plants, provides an excellent chance to discover new molecules with potent efficacy to potentially provide drug candidates (Kayser *et al.*, 2003; Al Nasr *et al.*, 2019b).

Euphorbia retusa Frossk and *Pulicaria undulata* L. are among the herbaceous flora of the Qassim Region, which has previously been subjected to scientific studies for biological activities and phytochemistry.

Euphorbiaceae is a family comprised of approximately 300 genera and 10,000 species, which are used in folk medicine to remove warts, for treating venomous bites and trichiasis, and for wound infection (Mengiste *et al.*, 2104). Phytochemically, *E. retusa* was visualized to afford a broad range of compounds that are mostly rich in carotenoids, fatty alcohol chains, sterols and essential fatty acids (Shaaban *et al.*, 2018). Biological investigations have shown that *Euphorbia* species possess antitumoral activity (Duarte *et al.*, 2006), antimicrobial activity (Murugan *et al.*, 2007), inhibition of HIV-1 viral infection, and antihyperglycemic and hypolipidemic activities (Hezareh, 2005).

P. undulata belong to the family Asteraceae, which includes approximately 80 species that are widely distributed in Europe, Asia and Africa (Boumaraf *et al.*, 2016). The plants of the genus *Pulicaria* have been used in traditional medicines to treat various ailments such as back pain, inflammation, menstrual cramps, intestinal disorders, dysentery, and diarrhea (Stavri *et al.*, 2008; Liu *et al.*, 2010). These plants possess various bioactivities such as cytotoxic, antipyretic, antioxidant, antispasmodic, antimicrobial, antihistaminic, analgesic, hepatoprotective, anti inflammatory, and nephroprotective effects (Ezoubeiri *et al.*, 2005; Yusufoglu, 2014; Foudah *et al.*, 2015). *P. undulata* is an aromatic medicinal plant that possesses large varieties of chemical substances, these chemical substances include alkaloids, flavones, flavonoids, sesquiterpene, lactones, diterpenes, triterpenes, naphthoquinones, anthocyanin, coumarin, catechins, isocatechins, and others (Christaki *et al.*, 2012; Chhetri *et al.*, 2015). Although some *Pulacarai* and *Euphobia* species have shown antiparasitic activities (Mengiste *et al.*, 2014; Mohamed *et al.*, 2017; Fadel *et al.*, 2018), this is the first investigation of *P. undulata* and *E. retusa* against *L. major* and *T. gondii* parasites.

MATERIALS AND METHODS

Plant material and preparation of extracts

Collection and processing of the plant materials
Whole plants of *E. retusa* and *P. undulata* were collected at December 2017 from Al Qassim region of Saudi Arabia, located in the center of the country (25° 32' 44" N, 43° 20' 40" E). Authenticated by Prof. Gamal E. Elghazali the major taxonomist from the College of Science and Arts in Ar Rass, Qassim University, Saudi Arabia, where the voucher specimens were deposited at the Department of Laboratory Sciences with reference numbers EURE041127111, PUUN041127112 respectively. The plant samples were washed by flushing with tap water dried under shade for 2 days, chopped to smaller pieces with a commercial blender and ground to fine powder using a Foss Tecator Cyclotec 1093 grinding mill. The fine powders were packed in airtight containers and stored at 4°C until ready for extraction (Al Nasr *et al.*, 2019b).

Preparation of the crude extracts
Approximately 200 g powder from the whole plants of *E. retusa* and *P. undulata* were soaked in 2 L of analytical grade methanol at room temperature overnight and allowed to stay in a mechanical shaker for 16 h for extract formation followed by 1 h of decantation. The extracts were then filtered using cotton in a normal funnel. The collected filtrates were pooled and evaporated to dryness under vacuum using a rotary vacuum evaporator IKA V 10. For each 500 ml batch from the methanol filtrate, the water bath temperature was set to 50°C, pressure 300 mbar and rotation at 60 rpm. The final sticky sludge of the extracts was recovered using methanol allowed to dry in petri plates in a circulatory hot air oven at 40°C for 6 h. The final form after complete drying was sealed with parafilm and stored in a refrigerator at 4°C until ready for further processing (Al Nasr *et al.*, 2019b).

Phytochemical fractionation of the plant crude extracts
The crude extracts were then suspended in distilled water and partitioned in different solvents on the basis of increasing polarity (using the solvent-solvent system of fractionation) and using a large separating funnel (5 L). The fraction yields for *P. undulata* methanolic crude included water (1500 mg), chloroform (700 mg), ethyl acetate (500 mg), n-butanol (1500 mg) and petroleum ether (Pet. Ether) (20 mg). For *E. retusa*, the yield was recorded as water (3500 mg), chloroform (500 mg), ethyl acetate (1000 mg), n-butanol (2000 mg) and Pet. ether (700 mg) (Han *et al.*, 2007).

Maintenance of Parasites and bioassays

Mice and parasite maintenance
Male and female SW and BALB/c mice (6-8) were obtained from pharmaceutical college, King Saud University, Saudi Arabia and maintained in specific-pathogen-free facilities. The research was conducted according to the rules of National Committee Bioethics, the research project was approved by Dean of Scientific Research, Qassim University (number cosao-bs-2019-2-2-1-5619).

Tachyzoites from the RH strain of *T. gondii* were obtained from Dr. Saeed El-Ashram (State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing, 100193, China). proliferated in SW mice (6-8 weeks age), and cryopreserved in liquid nitrogen at concentrations of 6×10^6 parasite/ml. Vero cells (ATCC® CCL81™, USA) were cultured in 75 cm² culture flasks in RPMI medium supplemented with 10% FBS (Sigma, Germany) and incubated at 37°C and 5% CO_2. *T. gondii* tachyzoites were maintained by serial passage in Vero cells grown in RPMI medium (Sigma, Germany) with 2% FBS (Jentzsch *et al.*, 2020).

The promastigote of *L. major* was isolated from a Saudi male patient in February 2016 and maintained at 26°C in Schneider's Drosophila medium (Invitrogen, USA) supplemented with 10% FBS and antibiotics in a tissue culture flask with weekly transfers. The promastigotes were cryopreserved in liquid nitrogen at a concentration of 3×10^6 parasite/ml. The virulence of *L. major* parasites was maintained by

passage in female BALB/c mice by injecting hind footpads with 1×10^6 stationary-phase promastigotes. After 8 weeks, *L. major* amastigotes were isolated from the mice. The isolated amastigotes were then transformed to promastigote forms by culturing at 26°C in Schneider's medium supplemented with 10% FBS and antibiotics. For infection, amastigote-derived promastigotes with less than five passages *in vitro* were used (Jentzsch *et al.*, 2020).

Evaluation of the antitoxoplasmal activity of plant extracts/fractions
Serial passage of the Vero cell line was used for the cultivation of *T. gondii* tachyzoites. Vero cells were cultured using complete RPMI 1640 medium with heat-inactivated 10% FBS in a humidified 5% CO_2 atmosphere at 37°C. The 96-well plates (5×10^3 cells/well in 200 µL RPMI 1640 medium) were used for the cultivation of the Vero cells and then incubated at 37°C and 5% CO_2 for one day followed by removal of the medium and washing the cells with phosphate buffer saline (PBS). Then, RPMI 1640 medium with 2% FBS containing tachyzoites of *T. gondii* were added at a ratio of 5 (parasites): 1 (Vero cells). After incubation at 37°C and 5% CO_2 for 5 h, the cells were washed with PBS and treated with RPMI 1640 medium containing extracts/fractions (dissolved in DMSO) (100, 33, 11, 3.7, 1.2, 0.4, 0.14 and 0.04 µg mL^{-1}), whereas RPMI 1460 medium containing DMSO (1%) without extracts/fractions or with atovaquone (ATO) (100, 33, 11, 3.7, 1.2, 0.4, 0.14 and 0.04 µg mL^{-1}) were treated as the negative and positive control, respectively.

After incubation at 37°C and 5% CO_2 for 72 h, the cells were stained with 1% toluidine blue after washing with PBS and fixation in 10% formalin. The cells were examined under an inverted photomicroscope to determine the infection index (number of cells infected from 200 cells tested) of *T. gondii*. The following equation was used for the calculation of the observed inhibition:

Inhibition (%) = (I Control – I Experimental)/(I Control) × 100
where "I Control" refers to the infection index of untreated cells and "I Experimental" refers to the infection index of cells treated with test compounds.

Then, the effects of the test extracts/fractions on parasite growth were expressed as EC_{50} (concentration that displayed 50% inhibition of parasites) values. The obtained EC_{50} values resulted from three independent experiments (Jentzsch *et al.*, 2020).

Activity of the crude extracts/fractions against *L. major* promastigotes
Promastigotes from logarithmic-phase cultures in phenol red-free RPMI 1640 medium with 10% FBS were suspended on 96-well plates to yield 10^6 cells mL^{-1} (200 µL/well), and a hemocytometer was used for parasite counting. The plant extract/fraction and reference drug Amphotericin B (AmB) were added to obtain the final concentrations (100, 33, 11, 3.7, 1.2, 0.4, 0.14 and 0.04 µg mL^{-1}). The negative control wells contained cultures with DMSO without extract/fraction, while AmB was used as the positive control with the same extract concentrations. The plates were incubated at 72 h to evaluate the anti-proliferative effect. The number of viable promastigotes were assessed by the colorimetric method using tetrazolium dye (MTT) and by measuring the reduction of the MTT component into an insoluble formazan product. This colored product is solubilized by adding a detergent solution to lyse the cells. The samples were analyzed by using an ELISA reader at 540 nm. The assay was performed in three independent experiments (Koko *et al.*, 2020).

Activity of plant extracts/fractions against *L. major* intra-macrophage amastigotes
The method of drug screening against *L. major* intracellular amastigotes was adapted from Calvo-Álvarez *et al.* (2012). Peritoneal macrophages from female BALB/c (6-8 weeks of age) were collected as described by Dos Santos *et al.* (2010). Then, 5×10^4 cells/well were seeded on 96-well plates in phenol red-free RPMI 1640 medium with 10% FBS for 4 h at 37°C and in a 4% CO_2 atmosphere to promote cell adhesion. The medium was discarded and washed by PBS, and a 200 µl containing *L. major* promastigotes solution (at a ratio of 10 promastigotes: 1 macrophage in phenol red-free RPMI 1640 medium with 10% FBS) was added to each well. Afterwards, the plates were incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere to allow infection and amastigote differentiation. Then, the infected macrophages were washed three times with PBS to remove the free promastigotes and overlaid with fresh RPMI 1640 medium containing extract/fraction and AmB at final concentrations (100, 33, 11, 3.7, 1.2, 0.4, 0.14 and 0.04 µg mL^{-1}) and then allowed to incubate at 37°C in a humidified 5% CO_2 atmosphere for 72 h. The negative control wells contained cultures with DMSO without extract/fraction, while AmB was used as a positive control with the same extract concentrations. The percentage of infected macrophages were microscopically evaluated after removing the medium by washing, fixation with methanol and staining with Giemsa. The assays were performed in three independent experiments (Koko *et al.*, 2020).

Toxicological evaluation of plant extracts/fractions *in vitro* using the MTT assay
The MTT assay was conducted for cytotoxicity evaluation of extracts/fractions. Briefly, Vero cells were cultured in 96-well plates (5×10^3 cells/well/200 µL) for 24 h in RPMI 1640 medium with 10% FBS and 5% CO_2 at 37°C. Cells were washed with PBS and treated with extracts/fractions for 72 h at varying concentrations (100, 33, 11, 3.7, 1.2, 0.4, 0.14 and 0.04 µg mL^{-1}) in the medium with 10% FBS. As a negative control, the cells were treated with the medium in 10% FBS. Thereafter, the supernatant was removed and 50 µL of RPMI 1640 medium containing 14 µL MTT (5 mg mL^{-1}) was added and incubated for 4 h. Afterwards, the supernatant was removed and 200 µL DMSO was added to dissolve the formazan. The FLUOstar OPTIMA spectrophotometer was applied for colorimetric analysis ($\lambda = 540$ nm). Cytotoxic effects were expressed by CC_{50} values (the concentration that displayed a 50% reduction in viable cells). The obtained CC_{50} values resulted from three independent experiments (Koko *et al.*, 2020).

Statistical analysis
EC_{50} and CC_{50} values were calculated by a linear regression equation via Microsoft excel. The SI (selectivity index) was calculated by dividing CC_{50} over EC_{50} for the inhibition result of each parasite. For comparing the differences between control negative means and the means of extracts/fractions student T test was used for the level of significant at $P \leq 0.01$.

RESULTS

Antitoxoplasmal activity
Table 1 indicates that the methanol extract of both *P. undulata* and *E. retusa* has good antitoxoplasmal activity with an EC_{50} of 12.7 and 5.6 µg mL^{-1}, respectively and significantly difference ($P \leq 0.01$). In the case of their fractions, the chloroform fraction showed the best activities with an EC_{50}

Table 1. Antitoxoplasmal activity of *P. undulata* and *E. retusa* methanolic extracts and their fractions against the *T. gondii* RH strain *in vitro*

Plant	Crude Extract and Fraction	Antitoxoplasmal Evaluation			
		Yield %	Antitoxoplasmal EC_{50} at µg mL^{-1}	CC_{50} of Vero cells at µg mL^{-1}	SI = CC_{50}/EC_{50}
P. undulata	Methanol	5	12.7 ± 3.1*	35.4 ± 6.6	2.8
	Pet. ether	0.1	7.3 ± 2.8*	27.6 ± 6.4	3.8
	Chloroform	0.35	1.4 ± 0.4*	17 ± 3.9*	12.1
	Ethyl Acetate	0.2	10.8 ± 2.6*	10.4 ± 2.3*	1.0
	n-Butanol	0.75	54.7 ± 11.2	33.9 ± 7.5	0.6
	Water	0.75	29.8 ± 5.3	6.1 ± 1.7*	0.2
E. retusa	Methanol	6	5.6 ± 1.9*	7.6 ± 2.1*	1.4
	Pet. ether	0.35	35.1 ± 6.7	24.9 ± 5.9	0.7
	Chloroform	0.1	8.7 ± 2.3*	17.4 ± 4.3*	2.0
	Ethyl Acetate	0.5	24.3 ± 4.6	29.3 ± 6.3	1.2
	n-Butanol	1	58.9 ± 12.4	22.9 ± 5.4	0.4
	Water	1.5	68.3 ± 14.9	29.2 ± 6.6	0.4
ATO			0.1 ± 0.02	9.3 ± 2.08	93.33

* P ≤ 0.01. The results mentioned in mean ± S.D.

Table 2. Antileishmanial activity of *P. undulata* and *E. retusa* methanolic extracts and their fractions against *L. major* promastigotes *in vitro*

Plant	Crude Extract and Fraction	Antipromastigote Evaluation		
		Antipromastigote EC_{50} at µg mL^{-1}	CC_{50} of Vero cells at µg mL^{-1}	SI = CC_{50}/EC_{50}
P. undulata	Methanol	6.3 ± 1.9*	35.4 ± 6.6	5.6
	Pet. Ether	7.3 ± 2.7*	27.6 ± 6.4	3.8
	Chloroform	3.9 ± 0.8*	17 ± 3.9*	4.4
	Ethyl Acetate	10.2 ± 2.6*	10.4 ± 2.3*	1
	n-Butanol	31.6 ± 4.5	33.9 ± 7.5	1.1
	Water	> 100	6.1 ± 1.7*	−
E. retusa	Methanol	22.0 ± 5.2	7.6 ± 2.1*	0.3
	Pet. Ether	82.1 ± 14.0	24.9 ± 5.9	0.3
	Chloroform	11.2 ± 3.7*	17.4 ± 4.3*	1.6
	Ethyl Acetate	73.6 ± 9.6	29.3 ± 6.3	0.4
	n-Butanol	89.1 ± 16.0	22.9 ± 5.4	0.3
	Water	> 100	29.2 ± 6.6	−
AmB		0.78 ± 0.09	7.4 ± 2.64	9.49

* P ≤ 0.01. The results mentioned in mean ± S.D.

of 1.4 and 8.7 µg mL^{-1}, respectively significantly difference (P ≤ 0.01). The activities of *P. undulata* fractions increased with the polarity increasing from Pet. ether to chloroform fractions and then decreasing from chloroform to n-butanol by increasing polarity. The fractions of *E. retusa* showed the same behavior, but the activity was not strong in comparison to the activity of *P. undulata*. The chloroform fraction of *P. undulata* showed a very strong SI of 12.1. The reference drug ATO gave EC_{50} 0.1 µg mL^{-1} and SI 93.3.

Antileishmanial promastigotes activity
Table 2 shows that the methanolic extracts of both *P. undulata* and *E. retusa* possess antileishmanial activity against *L. major* promastigotes with an EC_{50} of 6.3 and 22 µg mL^{-1}, respectively significantly difference (P ≤ 0.01). The antileishmanial activity of the fractions against *L. major* promastigotes decreases by increasing the polarity after chloroform for both plants. The chloroform fraction is most active for both *P. undulata* and *E. retusa* with an EC_{50} of 3.9 and 11.2 µg mL^{-1}, respectively significantly difference (P ≤ 0.01). The reference drug AmB showed EC_{50} 0.78 µg mL^{-1} and SI 9.49.

Antileishmanial amastigotes activity
Table 3 also revealed that chloroform fractions are still the most active for both *P. undulata* and *E. retusa* plants against *L. major* amastigotes with and EC_{50} of 3.8 and 18.5 µg mL^{-1}, respectively significantly difference (P ≤ 0.01). The ethyl acetate fractions of *P. undulata* also revealed antileishmanial activity against L. major promastigotes with an EC_{50} of 4.9 significantly difference (P ≤ 0.01), while all other fractions of *E. retusa* do not have any obvious activities against *L. major* amastigotes. The reference drug AmB revealed EC_{50} 0.46 µg mL^{-1} and SI 16.09.

DISCUSSION

This study indicates that *P. undulata* and *E. retusa* methanolic extracts have good antitoxoplasmal activity (their EC_{50} was 12.7 and 5.6 µg mL^{-1}) from primary screening, especially *E. retusa*, but after fractionation of the extracts the chloroform fraction of *P. undulata* was the best among all examined fractions with an EC_{50} of 1.4 µg mL^{-1}. This activity started from the Pet. ether fraction (EC_{50} 7.3 µg mL^{-1}) and then increased

Table 3. Antileishmanial activity of *P. undulata* and *E. retusa* methanolic extracts and their fractions against *L. major* amastigotes *in vitro*

Plant	Crude Extract and Fraction	Antiamastigote Evaluation		
		Antiamastigote EC_{50} at µg mL^{-1}	CC_{50} of Vero cells at µg mL^{-1}	SI = CC_{50}/EC_{50}
P. undulata	Methanol	−	35.4 ± 6.6	−
	Pet. Ether	25.2 ± 4.8	27.6 ± 6.4	1.1
	Chloroform	3.8 ± 0.7*	17 ± 3.9*	4.5
	Ethyl Acetate	4.9 ± 1.1*	10.4 ± 2.3*	2.1
	n-Butanol	72.6 ± 10.2	33.9 ± 7.5	0.5
	Water	29.5 ± 5.2	6.1 ± 1.7*	0.2
E. retusa	Methanol	−	7.6 ± 2.1*	−
	Pet. Ether	59.4 ± 9.5	24.9 ± 5.9	0.4
	Chloroform	18.5 ± 4.0*	17.4 ± 4.3*	0.9
	Ethyl Acetate	31.1 ± 5.6	29.3 ± 6.3	0.9
	n-Butanol	61.1 ± 9.7	22.9 ± 5.4	0.4
	Water	65.8 ± 8.9	29.2 ± 6.6	0.4
AmB		0.46 ± 0.07	7.4 ± 2.64	16.09

* $P \leq 0.01$. The results mentioned in mean ± S.D.

and reached the peak at chloroform (EC_{50} 1.4 µg mL^{-1}) and then started to decrease in the ethyl acetate fraction (EC_{50} 10.8 µg mL^{-1}). These results indicate the presence of active compound(s) eluted in the chloroform fraction. *P. undulata* is very rich in secondary metabolites, such as alkaloids, groups of terpenoids and flavonoids, all of them can be eluted in the chloroform fraction (Christaki *et al.*, 2012; Chhetri *et al.*, 2015). Previous research found low polar compounds with potent antitoxoplasmal activity such as terpenes (Khalid *et al.*, 2001) or alkaloids (Wei *et al.*, 2012; Maria *et al.*, 2013). The previous research supports and agrees with our results.

The chloroform fraction of *P. undulata* showed the most antileishmanial activity against both *L. major* promastigotes and amastigotes with EC_{50} of 3.9 and 3.8 µg mL^{-1}, respectively. These results indicate the presence of an active ingredient(s) in the chloroform fraction. In a previous study, an active indole alkaloid against *L. amazoniasis* was isolated from the chloroform fraction of *Peschiera australis* (Jan *et al.*, 2001). Much research has indicated the presence of active antileishmanial compounds in the chloroform fraction of methanolic extracts (Krishna *et al.*, 2007). These results also agree and support our findings.

The cytotoxicity evaluation against the Vero cell line indicates the safety of the chloroform fraction against *T. gondii*, *L. major* promastigotes and amastigotes due to SI values of 12.1, 4.4 and 4.5, respectively.

Although the crude methanolic extract of *E. retusa* provided very good activity against *T. gondii* with an EC_{50} of 5.6 µg mL^{-1}, all the other fractions revealed less activity. That may indicate a synergistic active ingredient distributed in different polarities. This type of relationship between fractions and compounds was previously detected (Sharma *et al.*, 2003).

In the present work the investigated reference drugs against both *T. gondii* and *L. major* promastigotes and amastigotes were gave EC_{50} values more strong in comparison to the used all plant extracts and fractions, that is due to their purity, while plant extracts and fractions contain multi number of compounds after isolation of the active ingredients we can expect more strong and potent results, those can be compared directly with the standard reference drugs.

CONCLUSION

In conclusion, the chloroform fraction of *P. undulata* is a very good candidate for isolation of antileishmanial and antitoxoplasmal drugs. Therefore, the further phyto-chemical analysis of active ingredient isolation is highly recommended.

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Conflict of interest
The authors declare that they have no conflict of interest.

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