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ORIGINAL ARTICLE

In vitro ovicidal activity of *Peganum harmala* seeds extract on the eggs of *Fasciola hepatica*

Mohammad Moazeni[1] · Zahra Sadat Saadaty Ardakani[1] · Mohammad Jamal Saharkhiz[2] · Jafar Jalaei[3] · Ali Asghar Khademolhoseini[1] · Shahab Shams Esfand Abad[1] · Amir Mootabi Alavi[1]

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Abstract *Peganum harmala* seeds extract has been previously reported to have antimicrobial and other medicinal properties. The aim of this study was to evaluate the ovicidal activity of the methanolic extract of *P. harmala* seeds against the eggs of *F. hepatica.* The phenolic compounds of the methanolic extract of *P. harmala* seeds were identified by HPLC analysis. Catechin, rutin, p-Coumaric acid, chloregenic acid and hesperetin were found to be the major phenolic compounds. *F. hepatica* eggs were collected from the gall bladder of naturally infected sheep. The eggs were exposed to two concentrations of *P. harmala* seeds extract (1 and 3 mg/mL) for 24 and 48 h. To investigate the effect of the *P. harmala* seeds extract on the miracidial formation, the treated eggs were incubated at 28 °C for 14 days. The results indicated that *F. hepatica* eggs were susceptible to the methanolic extract of *P. harmala* seeds. Following 24 h exposure of the eggs to *P. harmala* seeds extract with concentrations of 1 and 3 mg/mL, the miracidial formation reduced to 5 and 2.2 % respectively (compared with 60 % for the control group). Following 48 h of exposure of the eggs to *P. harmala* seeds extract with 1 mg/mL concentration, the miracidial formation reduced to 0.5 %. In this exposure time, no miracidial formation was observed in the eggs exposed to *P. harmala* seeds extract with concentration of 3 mg/mL. Therefore, the results of this study indicated that *P. harmala* seeds extract has high ovicidal activity against the eggs of *F. hepatica*. Accordingly, this extract may have the potential flukicidal activity against the immature and mature *F. hepatica*.

Keywords *Fasciola hepatica* · Miracidial formation · Ovicidal · Methanolic extract · *Peganum harmala* · Phenolic compounds

Introduction

Fasciolosis is an international parasitic disease of considerable economic and public health importance and *Fasciola hepatica* is the major cause of fasciolosis in man and domestic animals (Moazeni et al. 2010). The disease affects significantly the ruminant production by means of reducing the growth, conversion rate, milk production and quality and quantity of meat as well as decrease in reproduction (Martínez-Valladares et al. 2010). Effective strategies for the control of fasciolosis are mainly based on the use of drugs. Triclabendazole is the most widely used drug for the control of fasciolosis in ruminants and has until now proven to be the most effective flukicide against both mature and immature flukes (Brennan et al. 2007). Drug resistance has become a serious problem in veterinary medicine and an industrial threat in some regions of the world (Wolstenholme et al. 2004; Diab et al. 2009). Anthelmintic resistance continues to increase in geographic range, in the number of species affected and in the range of drugs involved (Sangster 1999). Resistance of *F. hepatica* against triclabendazole under practical conditions has been frequently reported (Overend and Bowen 1995; Lane 1998; O'Brien 1998; Mitchell et al. 1998; Fairweather and Boray

✉ Mohammad Moazeni
moazeni@shirazu.ac.ir

[1] Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz 71345-1731, Iran

[2] Department of Horticulture, College of Agriculture, Shiraz University, Shiraz, Iran

[3] Department of Pharmacology and Toxicology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

1999; Keiser et al. 2008; Fairweather 2009; Brockwell et al. 2014).

A number of strategies have been proposed in an attempt to preserve the efficacy of existing drugs and slow down the spread of resistance. Strategies involving drugs include the use of alternative anthelmintics, rotation of drugs from different chemical groups, selective treatment of animals, and use of drug combinations (Diab et al. 2009). New effective alternative treatment is extremely important in today's climate where species are becoming resistant and there has been a resurgence in the use of natural alternative therapies instead of synthetic pharmaceuticals that often have severe side effects (Harris et al. 2000), therefore, the investigation of chemical compounds from natural products is fundamentally important for the development of new anthelmintic drugs, especially in view of the vast worldwide flora (Kamaraj and Rahuman 2011).

Peganum harmala as a member of family Zygophyllaceae is an intriguing herb with a long history of medicinal use. It is widely grow in the most parts of the world including North and South America, Mexico, Africa and Asia. *P. harmala* is widely distributed in the Southern part of Iran too. It is known as "espand" in Persian and is famous for its medical uses as analgesic and antiseptic in folk medicine. Peganum seed contain carbohydrates, lipids, proteins, minerals, amino acids and alcoholic compounds as well as saturated fatty acids (Diba et al. 2011). Scientific reports show that the extract of *P. harmala* seeds has antimicrobial (Shahverdi et al. 2005), antibacterial (Nenaah 2010), antifungal (Diba et al. 2011; Ma et al. 2013), antiviral (Rashan et al. 1989), antiplasmodial (Astulla et al. 2008), antilieshmaneal (Mirzaie et al. 2007), antioxidant (Berrougui et al. 2006), antitumour (Lamchouri et al.1999), antinociceptive (Farouk et al. 2008) and hypoglycemic(Singh et al. 2008) properties. Since *P. harmala* seeds has a number of medicinal properties, the main goal of the current work was to evaluate the potential of the methanolic extract of this herbal plant to inhibit the miracidial development inside the eggs of *F. hepatica.*

Materials and methods

Experimental design

Fasciola hepatica eggs were exposed to *P. harmala* seeds extract for 24 and 48 h. Two concentrations (1 and 3 mg/mL) of the extract were used in this study. The experiments were performed at 37 °C (close to the normal body temperature for sheep). To determine the ovicidal activity of *P. harmala* seeds extract, the treated eggs were incubated at 28 °C for 14 days and miracidial formation was investigated under a light microscope. Nontreated eggs were considered as control group in each experiment. The experiments were performed in triplicate.

Preparation of *F. hepatica* eggs

Gallbladder of sheep naturally infected with *F. hepatica* were obtained from local abattoirs. The gallbladders were taken to the laboratory within 2 h in the sterile glass containers. The bile was aseptically transferred into glass cylinders and left to set for 30 min. The eggs settled in the bottom of the cylinders. The supernatant was then removed and the yielded eggs were washed several times using normal saline. The eggs were finally transferred into a dark glass container containing normal saline and stored at 4 °C for further use.

Preparation of *P. harmala* seeds extract

The fresh seeds of *P. harmala* were powdered mechanically using a commercial electric blender. To obtain the methanolic extract, 200 g of dry powder was added to 400 mL of pure methanol and mixed gently for 1 h using a magnetic stirrer. The obtained solution was left at room temperature for 24 h. The solution was stirred again and filtered using a filter paper (Grade 1 Whatman cellulose filter papers, Balstone, UK) and the solvent was then removed by evaporation in a rotary evaporator. The remaining semisolid material was then freeze-dried. The obtained residue was placed into a sterile and dark glass container and stored at 4 °C for further use (Moazeni and Nazer 2010). We obtained 15.08 g dried extract from 200 g of dried powder of *P. harmala* seeds.

Identification of phenolic compounds

For identification of the phenolic compounds of the extract, High Performance Liquid Chromatography (HPLC) analysis was carried out on a Agilent 1200 series (USA), equipped with a Zorbax Eclipse XDB-C18 column (10 cm × 5 μm i.d.; ×150 mm film thickness, RP), and a photodiode array detector (PAD). For preparing the injectable extract, 0.03 g of the dried residue of the plant extract was dissolved in 1 ml of methanol and the aliquots was filtered through a 0.2 μm membrane Millipore chromatographic filter and 20 μL of the solution injected into the HPLC system. The flow rate was set to 1 mL/min. The elution was monitored at 280 and 320 nm. Gradient elution was selected to achieve the maximum separation and sensitivity. The elution was performed by varying the proportion of solvent A (formic acid 1 % in deionized water) to solvent B (methanol (v/v)) as follows: methanol: formic acid 1 % (10:90), at 0 min; methanol: formic acid 1 % (25:75), at 10 min; methanol: formic acid 1 % (60:40), at

20 min and finally, methanol: formic acid 1 % (70:30), at 30 min. The total running time was 40 min. The column temperature was 30 °C.

Preparation of different concentration of *P. harmala* seeds extract

To prepare the *P. harmala* seed extract solution at 1 and 3 mg/mL concentrations, 10 and 30 mg of dried extract was dissolved in 10 mL of distilled water, respectively.

Ovicidal test

In this study, *F. hepatica* eggs were exposed to two concentrations of *P. harmala* seeds extract (1 and 3 mg/mL) for 24 and 48 h. In each experiment a drop of normal saline containing at least 500 eggs was added to a test tube containing 10 mL of *P. harmala* seeds extract. The tubes were then incubated at 37 °C for 24 and 48 h. At the same time, two containers containing at least 500 eggs with no exposure to extract were also incubated at 37 °C for 24 and 48 h as the control groups. Then, 9 mL of the upper part of the solution of each tube was removed with a pipette so as not to disturb the settled eggs.

Following several washes, the eggs of each tube were transferred into special small plastic containers (Supa Industries, Iran) containing 5 ml dechlorinated tap water. The containers were then incubated at 28 °C for 14 days. At the end of the incubation time, the eggs of each test and control groups were smeared on a manually scaled glass slide, covered with a cover glass, and examined under a light microscope. The counted eggs were divided into three groups: dead eggs, developing eggs(the eggs containing dividing cells) and the eggs containing live miracidia.

Statistical analysis

Differences between the rate of miracidial formation in the eggs treated with *P. harmala* seed extract and the untreated (control group) eggs were analyzed with Chi square test. Statistical analysis was performed with GraphPad InStat software and *P* values less than 0.01 were considered to be significant.

Results

HPLC analysis for identification of phenolic compounds from *P. harmala* seed extract, showed five phenolic compounds including catechin (11.52 mg/g), rutin (8.46 mg/g), p-Coumaric acid (6.03 mg/g), chloregenic acid (0.48 mg/g) and hesperetin (0.46 mg/g) (Table 1) as the main constituents.

Table 1 The phenolic components from *P. harmala* seeds extract using HPLC analysis

Phenolic component	(mg/g)	Retention time (min)
p-Coumaric acid	6.03	15.6
Rutin	8.46	12.6
Catechin	11.52	8.3
Hesperetin	0.46	22.4
Chloregenic acid	0.48	10.5

The results of the microscopic observations of the eggs exposed to *P. harmala* seed extract at different concentrations (1 and 3 mg/mL) and various exposure times (24 and 48 h) are presented in Table 2. While the miracidial formation rate for the eggs of control group was 60 %, following 24 h of exposure, the miracidial formation obtained for the eggs exposed to *P. harmala* seeds extract at concentrations of 1 and 3 mg/mL reduced to 5 and 2.2 % respectively. Following 48 h of exposure, the miracidial formation rate obtained for the eggs exposed to *P. harmala* seeds extract at a concentrations of 1 mg/mL reduced to 0.5 %. In this exposure time, no miracidial formation was observed in the eggs treated with *P. harmala* seeds extract at concentrations of 3 mg/mL. In two exposure times and two concentrations of the extract, the miracidial formation in the treated eggs reduced significantly compared to the control group ($P < 0.01$).

Discussion

Little information is available on the ovicidal effects of anthelminthic drugs. Ivermectin and artemether have been shown to have some ovicidal activity against *F. hepatica* eggs (Diab et al. 2009). The effect of some benzimidazole anthelmintics on nematode eggs has been well characterized. Fenbendazole is ovicidal against eggs of ruminant trichostrongylids, stomach worms in pigs, ascarids in chickens, and hookworms and whipworms in dogs (Panarella 2002). Albendazole has been shown to have a clear inhibitory effect on *F. hepatica* egg development, however, the most extensively used flukicidal compound, triclabendazole, did not affect the egg hatch, even in triclabendazole -susceptible flukes (Alvarez et al. 2009).

The rapid spread of triclabendazole resistance in veterinary medicine is an important motivation for fasciocidal drug discovery and development (Kirchhofer et al. 2011). In modern medicine, plants play a significant role since they possess various therapeutically important compounds having minimum side effects (Soliman and Fahmy 2011). On the other hand, development of parasite resistance to commercially available drugs have encouraged the search

Table 2 In vitro ovicidal effects of different concentrations of the methanolic extract of *P. harmala* seeds on *F. hepatica* eggs after 24 and 48 h of exposure

Exposure time (h)	Concentration	Experiment	Examined eggs	Dead eggs	Developing eggs	Eggs containing live miracidia	Miracidial formation (%)
24	1 mg/mL	1	652	271	362	19	2.9
		2	1637	486	1128	23	1.4
		3	2170	725	1264	181	8.3
		Total	4459	1482	2754	223	5
	3 mg/mL	1	898	554	297	47	5.2
		2	995	623	342	30	3
		3	1872	1458	409	5	1.9
		Total	3765	2635	1048	82	2.2
48	1 mg/mL	1	741	539	192	10	1.3
		2	841	599	242	0	0
		3	700	542	157	1	0
		Total	2282	1680	591	11	0.5
	3 mg/mL	1	1753	1541	212	0	0
		2	1090	962	128	0	0
		3	481	445	36	0	0
		Total	3324	2948	376	0	0
	Control	1	842	146	236	460	54.6
		2	555	70	145	340	61.3
		3	1490	368	187	935	62.8
		Total	2887	584	568	1735	60

for new active ingredients that are less toxic and more efficient. In this context, products of plant origin may be an effective alternative for the control of parasites (Nery et al. 2009). In addition, recent studies have revealed that the antihelminthic effect of some herbal plants may be equal to some widely used chemical antihelminthics (Moazeni et al. 2014a, b).

In the present study, we investigated the effect of the methanolic extract of *P. harmala* seeds on the miracidial formation in the eggs of *F. hepatica*. The results of our study showed that, the *P. harmala* seed extract at concentration of 1 mg/mL, after 24 and 48 h of exposure, can reduce the miracidial formation rate of *F. hepatica* eggs to 5 and 0.5 % respectively (compared with 60 % for the control group). However, with 3 mg/mL concentration of the extract, the above values reduced to 2.2 and 0.0 % respectively. Our recent study on ginger ovicidal activity revealed that a concentration of 1 mg/mL, after 24 and 48 h of exposure, reduced the miracidial formation rate of *F. hepatica* eggs to 52.28 and 47.82 % respectively (compared with 68.95 % for the control group) (Moazeni and Khademolhoseini 2016). When ginger extract was used at concentrations of 5 mg/mL, the mentioned values reduced to 1.16 and 0.0 % respectively. Therefore, the results of present study showed that, *P. harmala* seed extract has higher ovicidal activity in comparison with the methanolic extract of *Zingiber officinale*.

The ovicidal activity of herbal plants against many insect species (Obeng-Ofori and Reichmuth 1997; Obeng-Ofori et al. 1997; Tunc et al. 2000) and also *Haemonchus contortus* (Hounzangbe-Adote et al. 2001; Costa et al. 2002; Kamaraj and Rahuman 2011) have been previously evaluated.

The results of the present study revealed that the seed of *P. harmala* are rich in bioactive natural components such as catechin, rutin and p-Coumaric acid. These compounds maybe responsible for its biological effects. Scientific reports show that catechin has antioxidant (Iacopini et al. 2008; Zhu and Zhang 2014), antidiabetic (Zhu and Zhang 2014), antiviral (Daikoku et al. 2011) and anti-microbial (Chunmei et al. 2010) effects. Antioxidant (Iacopini et al. 2008) and antiviral (Han et al. 2015) activity of rutin has been previously reported. Antioxidant, anti-inflammatory, anticancer (Kadoma and Fujisawa 2008) and antiviral (Shimizu et al 1993) properties of p-Coumaric acid has also been previously demonstrated.

The mode of action of the phenolic components has been studied in the literature. For example, Ultee et al.

(2002) stated that the hydroxyl group and the presence of a system of delocalized electrons are important for the antimicrobial activity of phenolic compounds. Such a particular structure would allow the compounds to act as proton exchangers, thereby reducing the gradient across the cytoplasmic membrane. The resulting collapse of the proton motrice force and depletion of the ATP pool lead to eventual cell death.

Liu et al. (2013) determined the main and trace alkaloids in the seed extracts of *P. harmala* using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and high-performance liquid chromatography (HPLC).They found that Harmaline and harmine make more than 70% of the alkaloids. Diglycoside vasicine, vasicine, vasicinone, harmalol, harmol, tetrahydroharmine, 8-hydroxy-harmine, ruine, harmaline, and harmine were found to be the other alkaloids.

Total alkaloids of *P. harmala* seeds have been shown to have therapeutic effect on the bovine babesiosis and theileriosis (Fan et al. 1997), bovine natural tropical theileriosis (Mirzaei 2007) and ovine malignant theileriosis (Derakhshanfar and Mirzaei 2008).

The results of our study, clearly indicated that *F. hepatica* eggs are susceptible to the methanolic extract of *P. harmala* seed. Further studies are needed to determine the main compounds of *P. harmala* seed extract which are responsible for the ovicidal activities of this herbal plant.

In conclusion, development of helminth resistance to commercially available anthelmintic drugs, have encouraged the search for new compounds of plant origin with lower toxicity and higher efficacy. Our results revealed that, *P. harmala* seeds extract has high ovicidal activity against the eggs of *F. hepatica*. Accordingly, this extract offers an opportunity for new investigations. In fact, the potential flukicidal activity of *P. harmala* seed extract has not yet been investigated, and this extract, in addition to its ovicidal power, probably has some pharmacological activity against immature or mature *F. hepatica*. However, further studies should also focus on the in vivo efficacy of *P. harmala* seed extract and also its main alkaloids against the eggs, immature and mature flukes of *F. hepatica*.

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