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ORIGINAL PAPER

# Effects of *Artemisia annua* extracts on sporulation of *Eimeria* oocysts

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**Abstract** The present study aimed to compare the effect of different *Artemisia annua* extracts on sporulation rate of mixed oocysts of *Eimeria acervulina*, *Eimeria necatrix*, and *Eimeria tenella*. Three types of *A. annua* extracts including petroleum ether (PE), ethanol 96° (E), and water (W) extracts were prepared. Artemisinin, a sesquiterpene lactone endoperoxide derived from the *A. annua* analysis of each extract was done by high-performance liquid chromatography with ultraviolet detection (HPLC-UV). Fresh fecal samples containing three *Eimeria* species were floated and counted, and the oocysts were transferred into 50 tubes, each containing$10^5$ oocysts per milliliter. Five tubes were control. Each of the other 45 tubes contained one of three doses (1 part per thousand (ppt), 2 ppt, and 5 ppt) and one of three extracts (PE, E, and W extracts) with five replications. The tubes were incubated for 48 h at 25–29 °C and aerated. Sporulation inhibition assay was used to evaluate the activity of extracts. The results showed that the E and PE extracts inhibit sporulation in 2 and 5 ppt concentrations, but the W extract stimulates it in all concentrations. The proportions of oocyst inhibition relative to control were 31 % (5 ppt) and 29 % (2 ppt) for PE and 34 % (5 ppt) and 46 % (2 ppt) for E extract. Furthermore, many oocysts in PE and E groups were wrinkled and contained abnormal sporocysts. The proportions of sporulation stimulation relative to control were 22 % (5 ppt), 24 % (2 ppt), and 27 % (1 ppt) in W extract. Our study is the first to demonstrate that all types of *A. annua* extracts do not necessarily have a similar activity, and the interaction of all contents and their relative concentrations is an important factor for sporulation stimulation or inhibition. It seems, some parts of unmetabolized excreted PE and E extracts could inhibit oocyst sporulation and eventually affect infection transmission.

**Keywords** *Artemisia annua* · Extracts · Sporulation · *Eimeria* spp.

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## Introduction

Coccidiosis is regarded as one of the most important parasitic diseases of poultry industry, caused by *Eimeria* spp., belonging to the phylum Apicomplexa (Jang et al. 2007). It is estimated that the annual loss worldwide is more than 3 billion USD and for preventive medication, more than 300 million USD (Dalloul and Lillehoj 2006; McDougald 2013).

The oocyst of *Eimeria* is highly resistant and found wherever chickens are reared. Infected chickens shed oocysts in their droppings. The excreted oocyst is unsporulated and needs to be sporulated at a suitable temperature, relative humidity, and oxygen level. During sporulation, four sporocysts are formed within the oocyst, each containing two sporozoites (McDougald 2013; Kheysin 1972). Sporulation occurs in 24 to 48 h for most chickens, Eimerian species ideally (Edgar 1955; Waldenstedt et al. 2001). The sporulation rate of excreted oocyst is an important factor affecting infection pressure in a flock of chickens thus influencing the epidemiology of the infections (Waldenstedt et al. 2001).

The use of anticoccidial drugs over nearly the past six decades has provided the basis for the rapid growth of the poultry industry and produced high-quality white meat to

consumers. Although, prophylactic chemotherapy is still largely used in modern intensive poultry to control chicken coccidiosis; but with the increasing resistance of oocysts to the commonly used anticoccidial drugs (Khalafalla et al. 2011; Williams 2006), the high cost of the new drugs and the public health concerns of drug-treated meat demonstrate the urgent need of finding the least hazardous method to humans for controlling coccidiosis (Schubert et al. 2005; Molan et al. 2009; Williams 2006; Wunderlich et al. 2014).

*Artemisia annua* is a herb whose dried leaves have been used in traditional Chinese medicine for over two millennia. This plant grows naturally in the north of Iran (Klayman 1985; Bhakuni et al. 2001). The plant bioactive compounds include flavonoids, coumarins, steroids, phenolics, purines, lipids, aliphatic compounds, monoterpenoids, triterpenoids, and sesquiterpenoids such as artemisinin (Bhakuni et al. 2001; Brisibe et al. 2009). The anticoccidial effect of artemisinin in chickens was first reported by Allen et al. (1997). The medicinal effects of *A. annua* were reported as antimalarial, anticoccidial, antibacterial, anti-inflammatory, angiotensin-converting enzyme inhibitor, cytokinin-like, and antitumor (Bhakuni et al. 2001; Gholamrezaie Sani et al. 2013, Kayser et al. 2003).

This paper is an in vitro part of a project aiming to find alternative strategies to control coccidiosis in chicken by medicinal plants. Although a limited anticoccidial potential activity of *A.annua* in poultry is documented, there is no report about the external development of Eimeria influenced by incubation with different *A. annua* extracts (Allen et al. 1997). Therefore, the present study intended to compare the sporulation rate of mixed oocysts of *Eimeria acervulina*, *Eimeria necatrix*, and *Eimeria tenella* in the presence of different concentrations of *A. annua* extracts.

## Material and methods

### Collection of *A. annua*

*A. annua* was obtained from Firoozkooh in Mazandaran province in Iran. Plants were harvested in September, 2013 when flowers of the plants bud. Leaves of *A. annua* were stripped from the stem and dried for 1 month in a dark and ventilated room. The dried plants were powdered by mill and then sieved by a 4.75-mm mesh screen. Finally, they were stored at 4 °C for future application and analysis (Allen et al. 1997).

### Extracts preparation

Three types of extract (petroleum ether (PE), ethanol 96°(E), and water (W)) were prepared using the following method.

PE extract preparation: 30 g of the *A. annua* powder was dissolved in 250 ml petroleum ether and boiled at 30–50 °C for 8 h in a Soxhlet device. Then, the PE extract was dried in a vacuum. Finally, 2.4 g of extract was obtained (Liersch et al. 1986).

Ethanol extract preparation: 3600 g of the dried plant powder was moisturized with ethanol 96° for 15 min, placed in a percolator, and pressed followed by pouring with ethanol 96° on the top. The resulting solvent permeated to plant powder mass. When the first droplets exited, the valve was closed and ethanol was poured to 3–5 cm above the plant powder. The extract was discharged after 48 h at the speed of 5 ml/min (World Health Organization 2011).

Water extract preparation: 200 g of dried powder plant was mixed well with 2 l of boiling water in metal a dish, put on gentle flame for 2 h, and maintained at room temperature overnight. Finally, the extract was sieved (WHO 2011).

### Analysis of artemisinin

The extracts were analyzed by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) to measure artemisinin.

The prepared extraction was filtered and stored at −20 °C until analyzed by HPLC-UV PLATINblue Knauer model from Will Chrome Co, Germany. The standard artemisinin 97 % was prepared from Roth Co. Bavaria, Germany. The HPLC-UV system consisted of a Eurospher 100 C18, 25× 0.4 cm at 30 °C column, and wavelength detector was 260 nm. The gradient elution program was set at a flow rate of 1 ml/min starting with a $NaH_2PO_4$ 0.01 M and methanol mixture (20:80, *v*/*v*). The volume of a sample injected to the chromatograph was 20 μl at a flow rate of 1 ml/min. The artemisinin standard was used as external standard for plotting the calibration curve of the extract, and each analytical experiment was repeated at least three times.

### Experimental design and sporulation inhibition/stimulation assay

The fecal samples from infected broilers were obtained from poultry farms and transferred to a parasitology lab, Department of Pathobiology, Veterinary School, Shiraz University, Shiraz, Iran. The oocysts were identified as *Eimeria acervulina*, *E. necatrix*, and *E. tenella* through concentrating in saturated sodium chloride solution (specific gravity 36 g/100 ml) and sporulating in an aqueous solution of potassium dichromate 2.5 % (*W*/*V*) for 48 h (Molan et al. 2009; Conway and McKenzie 2007). Six 3-week-old broiler chickens were infected experimentally with $5\times10^5$ sporulated oocysts, and fecal samples were collected 8 days post infection, concentrated in saturated sodium chloride solution, counted using a hemocytometer, and applied for in vitro experiment.

An in vitro assay was done to evaluate the effect of different types and concentrations of *A. annua* extracts on the sporulation of *Eimeria* oocysts. In this assay, five million unsporulated oocysts in 50 tubes each containing $10^5$ mixed oocysts per milliliter were examined. The control group consisted of unsporulated oocysts in five tubes each containing $10^5$ mixed oocysts per milliliter in water. The test group was subdivided into three subgroups containing PE, E, and W extracts in three concentrations (1 part per thousand, 2 ppt, and 5 ppt) with five replications. The tubes were incubated for 48 h at 25–29 °C and aerated by an air pump. At the end of the incubation, the oocysts were washed twice in tap water. Then, the samples were stored at 4 °C until being counted (Molan et al. 2009). The number of sporulated oocysts was counted by hemocytometer method (Molan et al. 2009; Conway and McKenzie 2007).

### Statistical analysis

Data were analyzed by one-way ANOVA in a completely randomized design, and statistical means were compared using Duncan test at $P<0.05$.

## Results

The amount of artemisinin in *A. annua* was 500 ppm by petroleum ether (PE) method, and each sample was then evaluated as shown in Table 1 and Fig. 1.

The sporulation rate in different concentrations of extract is shown in Fig. 2. As shown in Fig. 2, 65.2 % of the oocysts were sporulated in the control group. The sporulation rates of oocysts in PE subgroups were 45.3, 43.2, and 60.6% for 5, 2, and 1 ppt concentrations, respectively. The sporulation rates were decreased in 5 and 2 ppt doses ($P<0.05$) compared to the control. In other words, sporulation inhibitions relative to control were 31, 34, and 7 %, respectively (Fig. 3). Similarly, the sporulation rates of oocysts in E subgroups were 46, 35.2, and 62 % in 5, 2, and 1 ppt doses, respectively. Compared to control, the sporulation rates were reduced significantly in 5 and 2 ppt doses ($P<0.05$), and sporulation inhibition relative to control was 29, 46, and 5 %, respectively (Fig. 3). No significant difference was observed among PE and E subgroups in both 5 and 2 ppt concentrates ($P>0.05$).

**Table 1** Results of extraction and analysis of *A. annua*

| Extract | Quantity of mealy extract of plant (%) | Quantity of artemisinin in mealy extract (%) |
|---|---|---|
| Petroleum ether | 8 | 0.61 |
| Ethanolic 96° | 10 | 0.23 |
| Watery | 20 | 0 |

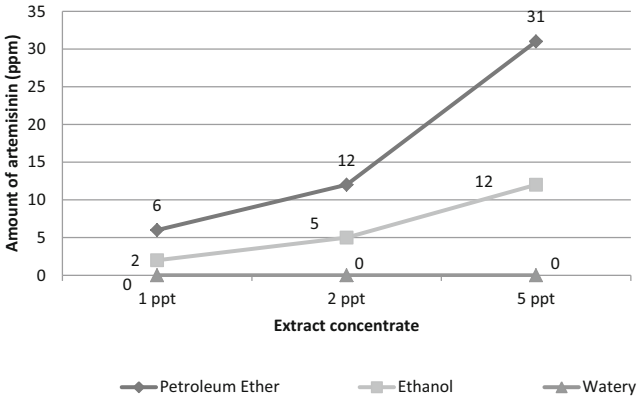


**Fig. 1** Amount of artemisinin in extracts concentrate

Furthermore, many oocysts of the PE and E groups (except for 1 ppt subgroups) were wrinkled and contained abnormal sporocysts, as evidenced by their abnormal sizes and shapes (data not shown), as compared to oocysts of the control group.

The sporulation rates of oocysts in W subgroups were 79.4, 80.8, and 82.6 % for 5, 2, and 1 ppt, respectively with a significant difference as compared with the control ($P<0.05$) (Fig. 2). Therefore, the sporulation stimulation relative to the control was 22, 24, and 27 % in 5, 2, and 1 ppt, respectively (Fig. 3), with no significant difference among the W subgroups.

## Discussion

Various methods including thin layer chromatography (TLC), high-performance liquid chromatography with UV detection (HPLC-UV), gas chromatography coupled with mass spectrometry (GC-MS), capillary electrophoresis coupled with UV detector (CE-UV), and enzyme-linked immunosorbent assay (ELISA) have been used to measure artemisinin (Mannan et al. 2010). We analyzed artemisinin by using HPLC-UV method for rapid, accurate, and cost-effective detection and quantification of artemisinin. This is normally the best method of artemisinin analysis (Mannan et al. 2010; Kim et al. 2001; Towler and Weathers 2007; Lapkin et al. 2009).

Oocyst sporulation is one of the most important factors affecting on the epidemiology of coccidiosis because poultry can only be infected when sporulated oocyst is ingested (Conway and McKenzie 2007; Molan et al 2009). *E. tenella* oocysts can survive in soil for up to 9 months (Marion and Wehr 1949). It seems that the oocysts excreted by treated birds may have an impaired sporulation. On the other hand, some parts of unmetabolized PE and E extracts are in close contact with oocysts on litter and could inhibit sporulation. Consequently, they affect the infection epidemiology (Reyna

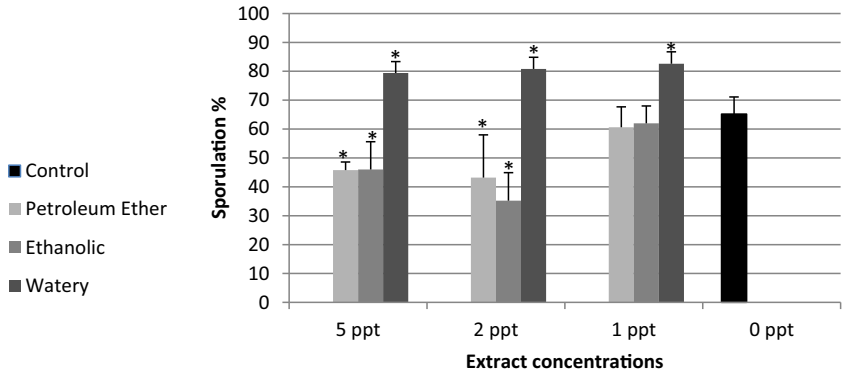


**Fig. 2** Percentage of sporulation in groups and subgroups

et al. 1983; Conway and McKenzie 2007; Molan et al. 2009; Wanamaker and Massey 2009).

After 3 days, 100 % of *E. acervulina* oocysts in litter are sporulated, while 30 % of them are already damaged (Williams 1995). The present study showed that PE and E groups (except for 1 ppt concentration subgroup) not only inhibit oocyst sporulation but also cause some morphological changes (shape and size). The mechanism is not found, but PE and E extracts of the plant may penetrate the oocyst wall and damage sporont in 2 and 5 ppt concentrations. Considering that the deformed oocysts are not infective, the impact on reducing the infective oocysts is, thus, doubled (McDougald 2013; Conway and McKenzie 2007).

The results of the present study showed that the type of extract is a key factor on oocyst sporulation of *Eimeria*. Both E and PE extract inhibited oocyst sporulation which was similar to what was reported by Goudarzi et al. (2005). The results of W extract of *A. annua* showed that sporulation was not only inhibited was but also stimulated in all concentrations. Therefore, although interaction of *A. annua* contents has an anticoccidial activity (Allen et al. 1997), interaction of different extracts composition has a variable effect on oocysts. It appears that the presence of artemisinin is one of the most important factors affecting sporulation inhibition. Considering that artemisinin is a sesquiterpene lactone with poor water solubility, the amount of artemisinin was zero in W extract, which was similar to the findings in previous research (Sweetman 2009). However, it seems there are other unknown ingredients that stimulate sporulation in W extract.

The present study showed that sporulation inhibition was done by PE and E extracts. For example, as Fig. 1 shows, the amount of artemisinin of E subgroup with 2 ppt concentration is less than that of PE subgroup with 1 ppt concentration significantly ($P<0.05$), but E subgroup with 2 ppt concentrations is the most effective on inhibition, and PE subgroup with 1 ppt concentration does not significantly affect inhibition of sporulation relative to control group ($P<0.05$). On the other hand, the subgroups had significant inhibitory effect on sporulation ($P<0.05$) and no significant differences among themselves; their amount of artemisinin was quite variable. Therefore, in addition to the types of extract, total contents and composition of extract are participating in sporulation inhibition or stimulation. It may be concluded that anticoccidial efficacy of artemisinin as a drug or feed additive requires further studies and more justification.

According to previous researches, the powder of *A. annua* has an in vivo anticoccidial activity (Allen et al. 1997). On the other hand, *Eimeria* species rapidly become resistant to chemotherapy. Thus, an alternative strategy should be used against avian coccidiosis (Conway and McKenzie 2007; Molan et al. 2009). The new approaches contain the use of natural products, probiotics, improved farm management, and modulation of the poultry immune system (Jang et al. 2007).

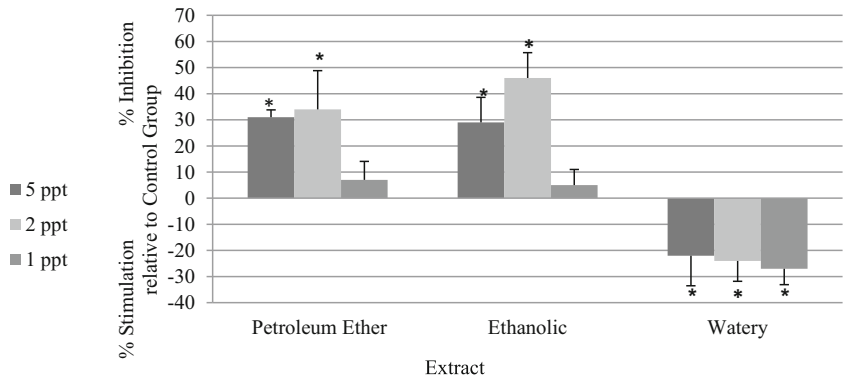


**Fig. 3** Percentage of stimulation or inhibition subgroups relative to control group

Therefore, more attention to this plant appears to be warranted in influencing the epidemiology of the infection.

In conclusion, our in vitro study showed that all types of *A. annua* extracts do not necessarily have a similar activity, and the external development of Eimeria may be influenced by different types of *Artemisia* extracts. PE and E extracts inhibited sporulation of *Eimeria* oocysts in 2 and 5 ppt concentrations. On the contrary, watery extract of this plant stimulated sporulation of *Eimeria* oocysts in 1, 2, and 5 ppt concentrations. PE and E extracts induced wrinkled and abnormal oocysts at the same concentration, while oocysts exposed to watery extract remained normal morphologically. So, all contents of extract, their relative concentration, the type of extract, and the total interaction of all contents of *A. annua* extracts and their relative concentrations are important factors on oocyst sporulation inhibition or stimulation. It seems that after oral administration of PE and E extracts, some parts of unmetabolized excreted drug could inhibit oocyst sporulation and eventually affect on infection transmission (Conway and McKenzie 2007; Molan et al. 2009; Wanamaker and Massey 2009). Further studies are needed to elucidate the exact mechanism of *A. annua* extracts on sporulation of *Eimeria* oocysts.

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

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