



# The Effectiveness of *Cinnamomum zeylanicum*, *Punica granatum* Flower and *Capsicum annum* Extracts Against *Parascaris equorum* Infective Larvae

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**Abstract**

Recent investigations have shown that plants with medicinal peculiarities as good alternative to anthelmintics for livestock. In this study, the anthelmintic effects of three medicinal herbs (*Cinnamomum zeylanicum*, *Punica granatum* flower and *Capsicum annum*) were screened *in vitro* against the infective larvae of *Parascaris equorum*. The recovered larvae of the parasite were exposed to four concentrations (50, 75, 100 and 125 mg/mL) of the extracts and then they examined for the viability at 0, 10, 20, 30 and 40 minutes after the challenge. The results revealed that all the concentrations of each plant extract had anthelmintic effects on *P. equorum* larvae. Also, the statistics indicated that there were significant interactions between the concentration of the extracts and time of exposure on the number of viable larvae. In addition, *C. annum* extract seemed to be a strong potency to kill larvae at all concentrations from the beginning of the experiment. These results confirmed that those herbal extracts possess good antiparasitic effects against infective larvae of *P. equorum* and thus could be considered in anthelmintic treatment strategies.

## Introduction

Anthelmintic resistance among intestinal nematodes of horses is a well-recognized problem worldwide. Of nematode parasites, the main indications for horse deworming are currently *Parascaris equorum* and cyathostomes, also called "small strongyles". *P. equorum* is a common intestinal nematode of foals under one year of age, but low burdens have been reported in horses up to four years old (Lyons et al., 2006). In addition to various milder gastrointestinal disturbances, *P. equorum* in some infection may even cause the host's death (Ryu et al., 2004).

Resistance against macrocyclic lactones (ivermectin and/or moxidectin), pyrantel salts and occasional resistance to piperazine has been reported in the Netherlands (Van Doorn et al., 2007), United States (Craig et al., 2007; Lyons et al., 2008), Denmark (Schougaard and Nielsen, 2007) and Italy (Veronesi et al., 2009). Resistance clearly involves the entire macrocyclic lactone class because sequential dosing of individual foals with ivermectin and moxidectin failed to

reduce egg counts after either treatment (Reinemeyer et al., 2010).

Although Pyrantel pamoate, Fenbendazole, and Oxibendazole have been used successfully to treat macrocyclic lactone-resistant populations of ascarids (Lyons et al., 2008; Schougaard and Nielsen, 2007; Slocombe et al., 2007), anthelmintic resistance issues and residues and toxicity problems (Gasbarre et al., 2001) have led to revival of interest in the validation of traditional veterinary practices (Ketzis et al., 2002; van Veen, 1997). Hence, there are numerous *in vivo* and *in vitro* surveys and scientific studies on validation of plants used as anthelmintics in the world (Alawa et al., 2003; Bizimenyera et al., 2006; Hussain et al., 2008; Iqbal et al., 2006a; Iqbal et al., 2006b; Jabbar et al., 2007). The aim of the present study was to assess the nematicidal activity of three methanolic extracts; *Cinnamomum verum*, Pomegranate (*Punica granatum* L.) and *Capsicum annum* L. on infective larvae of *P. equorum* larvae.

## Materials and Methods

### Parasites

Adult female worms of *P. equorum* were collected from the mass of parasites accumulated in the stomach and intestines of foals died due to an overwhelming disease and impaction by a ball of roundworm, brought to the large animal clinic, School of Veterinary Medicine, Shiraz University, southern of Iran. The collection and embryonation of the parasite eggs was performed as described by Burk et al. (2014), although with modifications. Briefly, the specimens were dissected and cut in tiny sections and then, grinded by a homogenizer, digested in acid pepsin solution (5 gram of pepsin powder in 1 L distilled water with 7 mL of hydrochloric acid in a proportion of 1/1 and then centrifuged. The supernatant removed and egg material was washed several times, placed in plates with shallow layers of sterile distilled water with 2.5% (w/v) aqueous potassium dichromate and were incubated for 4 weeks at 25-28°C with frequent aerations. The development of the eggs and larva formation was evaluated microscopically, until assured that larvae were developed in more than 90% of eggs. The egg solution was then stored at 4 °C until used.

### Hatching

In this study, the eggs were subjected to hatching process based on a previous method (Urban Jr. et al., 1981), although with some modifications. Briefly, a number of embryonated eggs were incubated at 37°C for 2 hours prior to hatching. Eggs were washed three times in normal saline via centrifugation for 5 min at 2000 rpm to remove the maintaining solution. The resulting suspension was washed 3 times by centrifugation at 2000 rpm for 5 min with 0.5 N NaOH, and then, 3 times in distilled water. The infective eggs were washed 5 times by centrifugation in normal saline at 2000 rpm for 5 min. The pellet of eggs was resuspended in tubes with about 10 ml of a solution of 6% sodium hypochlorite (as separate test tubes) at 37°C for 3 min and then, washed 5 times in normal saline. The hypochlorite-treated eggs were suspended in PBS (phosphate buffer solution), transferred to an Erlenmeyer flask containing a layer of glass beads (4-6 mm diameter) and agitated manually for approximately 1 minute. The whole suspension was transformed to a test tube, centrifuged at 1000 rpm for 5 min and the sediment examined microscopically to determine the proportions of the eggs hatched (as the ratio between the number of larvae to the number of embryonated eggs deposited), and larvae with appropriate motility. The obtained larvae were stored at 4°C until use.

### Plant materials and extraction

Methanolic extracts of *C. zeylanicum*, *P. granatum* flower and *C. annuum* were prepared as follows: After removing and collecting the plants, they were dried in the shade. After that, the dried leaves were ground into powder by electric blender, and then 100 g of each was dissolved in 400 mL of pure methanol separately. The solution was kept for 24 h at room temperature after mixing with a magnetic stirrer. The solvent was removed by evaporator system after filtering. Finally, the dry powder was obtained using lyophilization of the semisolid material. The obtained residue was placed in a sterile glass container and stored at 4°C till use (Moazeni and Nazer, 2010). For each extract, four concentrations (50, 75, 100 and 125 mg/mL) were prepared in sterile distilled water and incubated at 37°C for 1 hour before use.

### Nematicidal activity assay

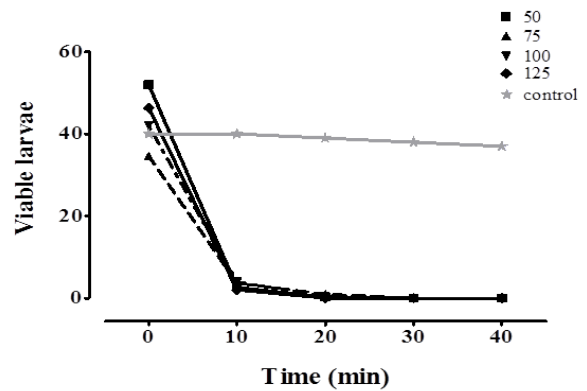
The recovered larvae were washed three times in normal saline and incubated at 37°C for 1 hour and placed under a light source for at least 15 minutes. The larvicidal effects of different extracts were evaluated based on Poné et al. (2011), albeit with modifications. Briefly, 30 µL of larvae solution containing of at least 30 larvae was transferred to a glass slide with round-bottomed well, counted, mixed with the same value of each plant extract at different concentrations (as treatment) or normal saline (as control) and covered by a cover slide. The mobility of larvae was noted by careful observation under a stereomicroscope (at 40x magnification) at 10, 20, 30, 40 minutes after adding the extracts. Those larvae with frequent whip-like movements were considered as viable. The number of immobilized larvae was determined and the percentage of the affected larvae (AL) was calculated as  $AL = \frac{T - D}{T}$ , where *T* is the total number of viable larvae deposited and *D* is the number of immobilized (as dead) ones. All experiments were conducted in three replicates.

### Statistical analysis

The larvicidal effects of different concentrations of each plant extract were evaluated by Repeated measures ANOVA followed by Tukey's test ( $P < 0.05$ ). Interactions between experimental time and treatment were also examined. All the analysis was performed with the IBM SPSS software package for Windows (Release 21, 2012, SPSS Inc.).

**Results**

Our data represented that all the concentrations of *C. zeylanicum* extract had anthelmintic effects on *P. equorum* larvae (Figure 1). The statistics also indicated that there was significant interaction between concentration and time on the number of viable larvae for the extract ( $P < 0.001$ ). In comparison with the control values, *P. granatum* flower had also marked nematocidal effects on larvae population at all concentrations ( $P < 0.02$ ) (Figure 2). In addition, there were a remarkable relation between the increase in the concentration and time on the number of viable larvae ( $P = 0.016$ ); however, the larvicidal effect obtained for the concentrations of 100 and 125 mg/mL were not significantly different ( $P > 0.5$ ). In this study, *C. annuum* extract evidenced a strong potency to kill viable larvae at all concentrations from the beginning of the experiment ( $P < 0.001$ ) (Figure 3). Although a significant relation was observed between the concentration and time of the experiment, the concentrations of 75, 100 and 125 mg/mL of *C. annuum* had similar effects on the parasite larvae ( $P > 0.5$ ).



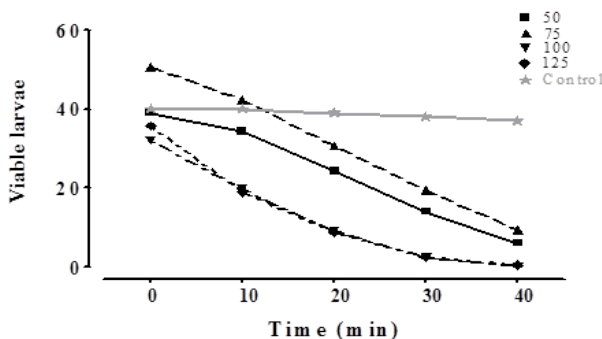
**Figure 3.** The effectiveness of *Capsicum annuum* extract on the viability of *P. equorum* larvae.

The concentrations of glucose, total protein, creatinine, hemoglobin, vitamin B12, ESR, count of lymphocyte and MCHC in the test group in the second samples were significantly higher: 16.4%, 10.78%, 17.8%, 11.7%, 9.5%, 14.7%, 22.2% and 10.5%, higher respectively ( $P < 0.05$ ) in relation to the first samples. Concentration of total bilirubin was 62.7% lower than in control group in; conjugated bilirubin concentration was 12.8% lower in the test group in the second samples in relation to the first samples.

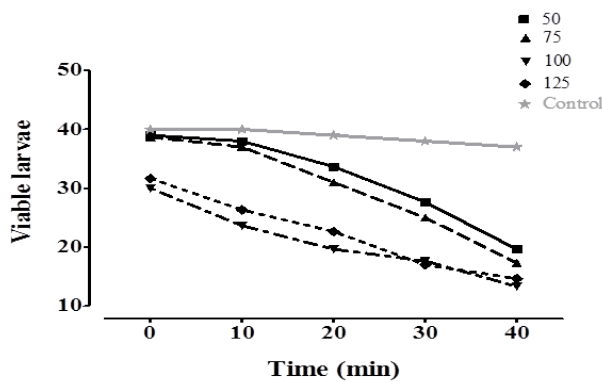
No significant differences were obtained in the contents of blood urea, serum iron, cholesterol, erythrocytes, leukocytes and hematocrit ( $P > 0.05$ ).

The concentrations of glucose in the blood serum in the reflex-1 and the reflex-2 groups in the test group in the second series of samples were significantly higher: 15.23% and 4.90% than in the control group, respectively; total protein concentration was 7.51% and 6.40% higher; creatinine concentration was 23.5% and 14.02% higher in, total bilirubin concentration was 56.15% and 17.8% lower; direct bilirubin concentration was 23.64% and 16.19% lower; vitamin B12 concentration was 8.4% and 3.39% higher; hemoglobin concentration was 8.2% and 2.1% higher; ESR was 16.3% and 9.6% higher; count of lymphocyte was 15.5% and 17.1% higher; MCHC was 10.05% and 1.04% higher.

The greatest increase of glucose concentration was achieved in the test group ( $5.99 \pm 0.4$  mmol/L;  $P < 0.05$ ). On the other hand, the smallest increase was observed in the reflex-1 and the reflex-2 groups, which only used BF-15 ( $5.27 \pm 0.83$  mmol/L in reflex-2 group in comparison with  $5.01 \pm 0.5$  mmol/L in the control group;  $P < 0.05$ ). The greatest increase of total protein concentration was achieved in the test group ( $77.13 \pm 15.2$  g/L;  $P < 0.05$ ). A significant increase was also observed in the reflex-1 and the reflex-2 groups ( $74.40 \pm$



**Figure 1.** The effectiveness of *Cinnamomum zeylanicum* extract on the viability of *P. equorum* larvae.



**Figure 2.** The effectiveness of *Punica granatum* flower extract on the viability of *P. equorum* larvae.

12.1 g/L and  $73.57 \pm 12.6$  g/L respectively in comparison with  $68.81 \pm 12.4$  g/L in the control group;  $P < 0.05$ ). Increased concentrations of serum creatinine occurred in all treated groups. However, the greatest increase was observed in the reflex-1 and the reflex-2 groups where was used only Haemobalans ( $157.11 \pm 29.5$  mmol/L in comparison with  $120.14 \pm 21.2$  mmol/L in the control group;  $P < 0.05$ ). The greatest increase in the concentration of vitamin B12 was in the test group ( $3942.39 \pm 107$  pg/mL in comparison with  $3567.27 \pm 93$  pg/mL in the control group;  $P < 0.05$ ). However, the increase of concentration of vitamin B12 was observed in all the treated groups. The greatest improvement in red blood cells was achieved with the use of Haemobalans (MCHC –  $311.52 \pm 13.2$  g/L in the test group;  $281.75 \pm 13.5$  g/L in the reflex-1 group,  $278.80 \pm 12.7$  g/L in the reflex-2 group and  $310.02 \pm 13.5$  g/L in the control group;  $P < 0.05$ ). The greatest improvement in white blood cells was achieved with the use BF-15 ( $24.18 \pm 1.6 \times 10^9$ /L lymphocytes in the test group;  $18.99 \pm 1.7 \times 10^9$ /L in the reflex-1 group;  $22.25 \pm 0.8 \times 10^9$ /L in reflex-2 group;  $18.80 \pm 2.1 \times 10^9$ /L in the control group;  $P < 0.05$ ).

None of the treated mares had significant histological changes of placenta structure. In the histological study, placenta of mares which were treated with Haemobalans held proliferation and vascular congestion of chorionic villi. It was associated with the angiogenic effect of the substance. Histological changes of placenta in the control group and the reflex-2 group included hyperemia and diapedesis into chorionic villi, protein degeneration of syncytiotrophoblast, shortened villi. Trophoblast layer was thinned; trophoblast cells did not assemble into functional knots and produced a small amount of syncytium. There were edematous connective tissues with collagen destructors and vasodilatation of internal vessels. The results of histological study are shown in figures 1 and 2.

The proof of adaptogenic effects of the integrated treatment is based on the determination of mortality rate during the antenatal period, which is shown in Table 3. The acquired data are not statistically sufficient; however, they demonstrate the tendency of the increase of the vital ability of foals in the antenatal period due to the integrated treatment

### Discussion

The application of Natural Anthelmintic Products (NAPS) is a new alternative method for the control of helminths infection worldwide, especially against parasitic nematodes (Hussain et al., 2008). There is a lot of evidence that NAPS are very effective and useful as

anthelmintic drugs (Costa et al., 2006; Jabbar et al., 2007; van Veen, 1997). These substances are commonly used because they are generally inexpensive, environmental friendly and safe to both man and animals (Hussain et al., 2008).

Our data showed that different concentrations of *C. zeylanicum*, *P. granatum* flower and *C. annuum* methanolic extract had a strong potency for damaging *P. equorum* larvae; however *C. annuum* was more effective than others since all the concentrations showed about highest nematicidal activity after the first 10 minutes. Furthermore, the significant correlation between increased concentrations of methanolic extracts and fatality rate of *P. equorum* larvae, which is in agreement with previous studies (Bizimenyera et al., 2006; Costa et al., 2006; Iqbal et al., 2006b). In line with our data, Rabiul et al. (2011) represented the efficacy of aqueous extract of *Cinnamomum camphora* against adult Roundworm (*Ascaridia galli*) and Tapeworms (*Railletina spiralis*) in a dose-dependent manner. In that study, the maximum efficacy was at 50 mg/mL concentration while this plant extract exhibited more potent activity at lowest concentration (10 mg/mL) against (roundworm) *Ascaridia galli*. Similarly, Fichi et al. (2007) revealed the effects of essential oil of *Cinnamomum zeylanicum* against *Psoroptes cuniculi*. These results combined with our data could obviously show that *Cinnamomum* species have a good prospect for the treatment of infections with helminthes in adult and larvae stages.

In our work, the methanolic extract of *P. granatum* (pomegranate) flower represented a significant reduction effect on the parasite larvae. Pomegranate belonging to family of Punicaceae widely cultivated in the Mediterranean region. It has known as an anti-diarrheal, antiparasitic agent for treatment of ulcers, diuretic, and antibacterial for the treatment of ulcers. Corroborating our data, some reports have been emphasized on the effect of pomegranate on the parasitic agents. Al-Mathal et al. (2012) indicated the effectiveness of Pomegranate in murine model experimentally infected with *Cryptosporidium parvum*. In their study, *P. granatum*-treated mice had significantly reduced oocyst shedding, higher mean weight gain, significant improvement of histological architecture of villi and reduced numbers of *C. parvum* trophozoites. In another in vivo study, Dkhil (2013) investigated the effects of methanolic extract of pomegranate peel on groups of mice experimentally infected with *Eimeria papillata*. In this experiment, the oocyst output was significantly decreased to 50 % in pomegranate-treated mice. Also, pomegranate extract caused a great diminish in body weight loss of infected mice, significant increase in the number of goblet cells

within the infected villi and a marked decrease in the number of apoptotic cells due to *E. papillata* infection. In addition, pomegranate was able to exert a significant anthelmintic effect on live adult *Allolobophora caliginosa* worms at different concentrations (100, 200 and 300 mg/mL). These data demonstrated that *P. granatum* is a promising anti-coccidial agent. In addition to this study, some previous works also indicated that *P. granatum* has marked effects on cestode, nematode (Abdel-Ghaffar et al., 2011) and protozoan (Dell'Agli et al., 2009) infections. Moreover, ethanol extracts of the *P. granatum* has revealed different antibacterial activity against *Pasteurella haemolytica*, which affected sheep, and had shown completely antibiotics resistance (Al Laham and Al Fadel, 2013). The antibacterial effect has been attributed to phenolic compounds which could increase the concentration of organic acids (Orak et al., 2011).

In the present study, *C. annuum* showed an interesting negative effect on the viability of *P. equorum* larvae. Although, reports on the potency of *C. annuum* for eliminating the infective agents is rare but it seems that more in vitro and in vivo studies should be conducted to assess the protective activity of this plant against helminths infections in different hosts.

Based on present data and also previous studies on *C. zeylanicum*, *P. granatum* flower and *C. annuum* we can suggest that this herbal extracts can be very useful as NAPS.

### Conclusion

Our in vitro study confirmed *C. annuum*, *C. zeylanicum*, and *P. granatum* flower are effective and useful as anthelmintic agents, significantly. However, *C. annuum* was more effective and its application can be considered as a simple, effective, safe and cheap treatment for controlling nematode infection and can be a good candidate to treatment of parasitic nematode in animal. Also to obtain more details, field efficacy on studied plants should be necessary to gain more confirmation and details.

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