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Research Article

# A STUDY ABOUT ANTHELMINTIC EFFECT OF *PUNICA GRAMATUM* L BARK ON VETERINARY ENDOPARASITES

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**ABSTRACT**

Objective: The present research was performed to evaluate the anthelmintic activity of *Punica gramatum* L plant bark on veterinary endoparasites.

Method: We used 4 methods to extract the bark of *Punica gramatum* L plant, including using water, water and heat, water with previous soak in NaOH 5 % and water with previous soak in $CH_3COOH$ 5 %. Those solutions then were tested with animal endoparasites, including porcine ascarids, porcine cestodes, chicken ascarids and chicken trematodes at the concentrations of 20, 10, 5, 2.5 and 1.25 %. Their anthelmintic activity was evaluated through the lethal time by which the treatment of extracts induced the death of tested parasites. Result: bark of *Punica gramatum* L plant possesses anthelmintic efficacy with animal endoparasites. All of extracts at 5 % were able to kill 100% of experimental parasites within the tested time (360 minutes). Extraction with $CH_3COOH$ 5 % had the best effect, with the shortest necessary treatment time in all of tested concentrations with 4 experimental endoparasites. Conclusion: *Punica gramatum* L plant bark has *in vitro* anthelmintic on porcine ascarids, porcine cestodes, chicken ascarids and chicken trematodes, and follow-up researchs are necessary to assess the *in vivo* effect.

**Keywords:** *Punica gramatum* L, bark, extract, anthelmintic effect, endoparasites

## INTRODUCTION

Most of the veterinary endo-parasite infections in animals are chronic. Infestation with endoparasites in digestive tracts can cause significant economic losses because of long-term nutrition absorbance hazard lead to the loss of weight and productivity, and also increase the prevalence of many other diseases due to the animal immuno-depression [1]. The development of resistant strains of parasites to currently available anthelmintic drugs has been reported [2], [3], [4]. The continuous and long-term reliance on a small range of compounds has led to the development of drug resistance in many helminthic strains [5]. In addition, the increased public awareness for synthesis drug residues in consumed animal products which possessed potential to hazard human also enforces the search for alternative therapy. There is an increasing demand towards natural anthelmintics [6]. In the effort find alternatives for modern products, medicine plants serves as the most potential one [7]. Medicinal plants have been used to treat paratisism in animals for hundreds of years [8]. According to Gerold Rahmann and Hannah Seip [9], 31 medicine plants and 8 mixtures of plants and other components have been considered as possible alternative althelmintic for endoparasites. The *in vitro* and *in vivo* althelmintic properties of many plants were also reported [10], [11]. Food supplements like papaya, cinnamon, turmeric, asafetida, long pepper saffron, Moringa, bitter guard and fresh juice of pine apple also have anthelmintic property [12]. In Vietnam, medicine plants have been considered as a traditional therapy to control animal parasites with the advantages of less side-effect and inexpensive cost [13]. Following Vietnamese ethnic experiences, the bark of *Punica gramatum* L has been known to have anthelmintic property and is usually applied to treat endoparasites for both human and animals, but its usage is only based on handed down knowledge [14], [15]. Therefore, it is necessary to research about this plant to verify the anthelmintic effectiveness and to propose the allopathic phytotherapeutical approach for its application.

## MATERIAL AND METHODS

### *The collection of endoparasites*

experimental parasites, including porcine ascarids, porcine cestodes, chicken ascarids and chicken trematodes were collected in the local slaughter houses which are located near the laboratory (Vang market slaughter house and Da Ton market slaughter house, Trau Quy, Gia Lam, Hanoi). Samples were kept in physiological saline solution (PSS) to bring back to laboratory, and the authentify was performed in laboratory under the supervision of Associate professor Bui Thi Tho, department of pharmacology faculty of Veterinary Medicine, Hanoi university of agriculture. The tests with extracts were then started within 2 hs from the parasite collection time.

### The collection and extraction of Punica gramatum L bark

The bark of the *Punica gramatum* L plants was collected on the period from February to April because following the advices of Vietnamese herbalists, spring season is the favorable period for this herb collection. The bark was then washed, preliminarily dried in the shadow for 3 to 4 sunny days, futher dried in the oven at 50 $^oC$ for 4 hs before being ground into powder with particle size less than 1 mm. The herb samples were kept in airtight plastic bags in the dried places for maximum 6 months before using.

We performed the extraction following the methods which was usually used for herb in Vietnam [16], [17]. Four methods, including (1) water with previous soak in $CH_3COOH$ 5 %, (2) water with previous soak in NaOH 5 %, (3) steeping in water and (4) boiling in water were performed to extract. For (1) and (2) methods, 50 g bark powder was wetted with 15 ml $CH_3COOH$ 5% or 15 ml NaOH 5 % for 1 h before adding 100 ml of distilled water (DW) and further left for

23 hs. For (3) method, 50 g powder was steeped in 115 ml DW for 24hs. For (4) method, 50 g powder was boiled in 115 ml DW for 15 ms. Collected solutions were then filtered through 2 layers of cheese cloths. The filtrates were adjusted with DW to make 100 ml. HCl or NaOH 50 % was used to adjust the pH of the solutions to be from 6.9 to 7.1. The extracts were called acid-DW, bazo-DW, DW and hot-DW extracts, in accordance with the solvents which were used in (1), (2), (3) and (4) methods, respectively. The initial extracts were considered as 50 % (meaning 50 g in 100 ml), and were diluted by PSS to test at 20 %, 10 %, 5 %, 2.5 % and 1.25 % to test with endoparasites.

**The extract treatment and the measurement of lethal time**

The tests were conducted following the methods of Nguyen Nhu Vien [17]. In the experimental groups, each of 10 parasites was put in petri dish that contained the extracts at different concentrations. The PSS was used in control groups. All of tested parasites were observed to survive for at least 24 hs in the PSS. We checked the paralysis of individual worms in experimental group every minute during the 360 ms of experiment. Paralysis was said to occur when the parasites lost their motility and do not revive even in the normal PSS.

The time that induced the death of 50 % experimental parasites, called lethal time 50 (LT50) and the time that induced the death of 100 % experimental parasites, called lethal time 100 (LT100), was calculated from the linear regression computerized between the time and the percentage of parasites died at that time.

***Statistic analyse***

data was expressed as mean ± standard error (Mean ± SE). Data were analyzed using the Statcel software (Yanai Hisae, Laboratory of mathematics, Faculty of Science, Saitama University, 1998). The result was considered significant at probability value less than 0.05 ($p < 0.05$).

**RESULTS**

In order to evaluate the anthelmintic efficacy of *Punica gramatum L* bark, we measured and calculated the LT50 and LT100 of different extracts. The results were shown in Table 1.

**Table1: The LT50 and LT100 of extracts of *Punica gramatum* L plant bark on animal endoparasites**.

| | | LT50 | | | | LT100 | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Extract** | **Conc.** | **Porcine ascarides** | **Porcine cestodes** | **Chicken ascarids** | **Chicken trematodes** | **Porcine ascarides** | **Porcine cestodes** | **Chicken ascarids** | **Chicken trematodes** |
| Acid-DW | 20% | 30 ± 2.3 $^{a}$ | 15 ± 3.3 $^{a}$ | 20 ± 5.1$^{a}$ | 10 ± 2.1 $^{a}$ | 65 ± 5.3 $^{a}$ | 25 ± 3.3 $^{a}$ | 50 ± 2.9 $^{a}$ | 20 ± 2.2 $^{a}$ |
| | 10% | 45 ± 4.4 $^{b}$ | 25 ± 2.6 $^{b}$ | 25 ± 2.1$^{b}$ | 12 ± 0.9 $^{a}$ | 70 ± 8.9 $^{b}$ | 30 ± 2.4 $^{b}$ | 60 ± 5.1 $^{b}$ | 25 ± 2.3 $^{ab}$ |
| | 5% | 60 ± 4.4 $^{c}$ | 35 ± 2.6 $^{c}$ | 50 ± 6.5 $^{c}$ | 15 ± 1.1 $^{b}$ | 85 ± 8.2 $^{bc}$ | 50 ± 2.5 $^{c}$ | 110 ± 9.9 $^{c}$ | 30 ± 2.5 $^{b}$ |
| | 2.50% | 75 ± 8.7 $^{d}$ | 37 ± 3.5 $^{c}$ | 115 ± 9.5 $^{d}$ | 20 ± 1.8 $^{c}$ | 95 ± 9.9 $^{d}$ | 55 ± 4.7 $^{c}$ | 160 ± 10.1 $^{d}$ | 38 ± 3.4 $^{c}$ |
| | 1.25% | 110 ± 8.9 $^{d}$ | 50 ± 3.3 $^{d}$ | 145± 11.0 $^{e}$ | 27 ± 2.2 $^{d}$ | 160 ± 12.3 $^{e}$ | 75 ± 10.1 $^{d}$ | 225 ± 8.1$^{e}$ | 45 ± 3.2 $^{d}$ |
| Bazo-DW | 20% | 50.3 ± 5.7 $^{a}$ | 20.2 ± 2.4 $^{a}$ | 30.4 ± 3.0 $^{a}$ | 15.2 ± 4.1 $^{a}$ | 70 ± 2.3 $^{a}$ | 40 ± 3.4 $^{a}$ | 65 ± 4.4 $^{a}$ | 21 ± 5.1 $^{a}$ |
| | 10% | 55 ± 4.6 $^{ab}$ | 25 ± 2.1$^{a}$ | 45 ± 4.1 $^{b}$ | 17 ± 0.6 $^{a}$ | 80 ± 7.9 $^{b}$ | 50 ± 5.4 $^{b}$ | 80 ± 10.3 $^{b}$ | 30 ± 2.1 $^{b}$ |
| | 5% | 75 ± 5.3$^{b}$ | 35 ± 3.1$^{b}$ | 70 ± 8.8 $^{c}$ | 20 ± 2.1$^{b}$ | 95 ± 9.8 $^{bc}$ | 55 ± 6.1 $^{bc}$ | 130 ± 12.1 $^{c}$ | 35 ± 3.5 $^{c}$ |
| | 2.50% | 80 ± 7.7 $^{c}$ | 40 ± 3.1$^{c}$ | 145 ± 10.9 $^{d}$ | 25 ± 0.9 $^{bc}$ | 110 ± 10.0 $^{c}$ | 70 ± 10.3 $^{c}$ | 190 ± 12.0 $^{d}$ | 45 ± 5.4 $^{d}$ |
| | 1.25% | 90 ± 7.5 $^{d}$ | 50 ± 2.1$^{d}$ | 155 ± 12.7 $^{d}$ | 30 ± 3.1$^{c}$ | 170 ± 9.9 $^{d}$ | 120 ± 13.9 $^{d}$ | 235 ± 7.0 $^{e}$ | 50 ± 3.3 $^{d}$ |
| DW | 20% | 80 ± 7.8 $^{a}$ | 30 ±3.2 $^{a}$ | 155 ± 10.3 $^{a}$ | 12 ± 1.9 $^{a}$ | 110 ± 9.6 $^{a}$ | 45 ± 4.9 $^{a}$ | 230 ± 4.34 $^{a}$ | 21 ± 2.3 $^{a}$ |
| | 10% | 95 ± 5.8 $^{b}$ | 35 ± 3.2 $^{a}$ | 160 ± 12.3 $^{a}$ | 15± 1.2 $^{a}$ | 120 ± 10.2 $^{b}$ | 55± 4.9 $^{b}$ | 265 ± 4.3 $^{b}$ | 28 ± 1.9 $^{b}$ |
| | 5% | 115 ± 10.8 $^{c}$ | 45 ± 5.9 $^{b}$ | 255 ± 4.2 $^{b}$ | 17 ± 2.3 $^{a}$ | 135 ± 10.2 $^{b}$ | 70 ± 7.6 $^{c}$ | 325 ± 3.9 $^{c}$ | 27 ± 2.2 $^{b}$ |
| | 2.50% | 120 ± 11.2 $^{c}$ | 55 ± 4.2 $^{c}$ | 350 ± 2.3 $^{c}$ | 35 ± 3.2 $^{b}$ | 170 ± 9.8 $^{c}$ | 80 ± 9.9 $^{c}$ | (*50 ± 5.8) | 45 ± 3.3 $^{c}$ |
| | 1.25% | 140± 12.0 $^{d}$ | 70 ± 2.5 $^{d}$ | (-) | 35 ± 3.4 $^{b}$ | 190 ± 10.3 $^{d}$ | 135 ± 12.0 $^{d}$ | (*30 ± 4.9) | 55 ± 4.1 $^{c}$ |
| Hot DW | 20% | 75 ± 3.4 $^{a}$ | 18 ± 1.8 $^{a}$ | 118 ± 9.9 $^{a}$ | 15 ± 1.3 $^{a}$ | 90 ± 8.7 $^{a}$ | 35 ±3.4 $^{a}$ | 213 ± 14.0 $^{b}$ | 30 ± 3.3 $^{a}$ |
| | 10% | 85 ± 6.0 $^{b}$ | 20 ± 2.3 $^{a}$ | 135 ± 9.9 $^{b}$ | 20 ± 2.1$^{b}$ | 115 ± 9.6 $^{b}$ | 40 ± 3.2 $^{a}$ | 175 ± 11.8 $^{a}$ | 40 ± 2.9 $^{b}$ |
| | 5% | 90 ± 7.6 $^{b}$ | 20 ± 2.5 $^{a}$ | 178 ± 10.1 $^{c}$ | 25 ± 2.0 $^{bc}$ | 140 ± 1.3 $^{bc}$ | 55 ± 5.9 $^{b}$ | 238 ± 5.0 $^{c}$ | 35 ± 3.3 $^{a}$ |
| | 2.50% | 110 ± 9.9 $^{c}$ | 30± 2.3 $^{b}$ | 195 ± 9.7 $^{c}$ | 30 ± 2.5 $^{c}$ | 170 ± 11.9 $^{c}$ | 70 ± 8.7 $^{c}$ | 255 ± 7.3 $^{c}$ | 40 ± 2.9 $^{b}$ |
| | 1.25% | 125 ± 9.7 $^{c}$ | 45± 3.2 $^{c}$ | - | 35 ± 2.9 $^{c}$ | 215 ± 9.7 $^{d}$ | 115 ± 9.5 $^{d}$ | (*40 ± 7.8) | 60 ± 3.9 $^{c}$ |
| Control | No parasites was death in control group | | | | | | | | |

**(*n) means that there was only n % of experimental parasite died after 360 ms of observation. (-) means that there was no experimental parasites died after 360 ms of observation. Means of the same extract at different concentrations which have different superscripts (a, b, c, d, e) are significantly different ($p < 0.05$) by one-way ANOVA and *Post – hoc* Fisher's least significant difference test. Bold letters indicates the shortest LT 50 and LT 100 with different endoparasites**

From Table 1, we see that the extracts of *Punica gramatum* L plant bark possessed the anthelmintic effect and this effect is dose-dependent in all of tested extracts, because following the dilution of the concentration, the LT50 and LT100 of each extract to each endoparasite was significantly longer. We also observed that at 5 %, all of the extracts were able to killed all tested parasites with the LT100 from 20 ± 2.2 ms (acid-DW extract 20 % to chicken trematodes) to 325 ± 3.9 ms (DW extract 5% to chicken ascarids). Acid-DW extract at 20% had the best anthelmintic effects, shown by the lowest LT50 and LT100 values to all of tested endoparasites, including porcine ascarides (40 ± 2.3 ms and 95 ± 5.3 ms), porcine cestodes (15 ± 3.3 ms and 25 ± 3.3 ms), chicken ascarids (20 ± 5.1 ms and 50 ± 2.9 ms) and chicken trematodes (10 ± 2.1 ms and 20 ± 2.2 ms).

In order to evaluate the effect of different extracts, we compared the LT100 values at the concentrations of 20 %, 10 % and 5 %, the concentrations which were able to kill all of experimental parasites within tested time (360 ms). The results are shown in Figure 1.

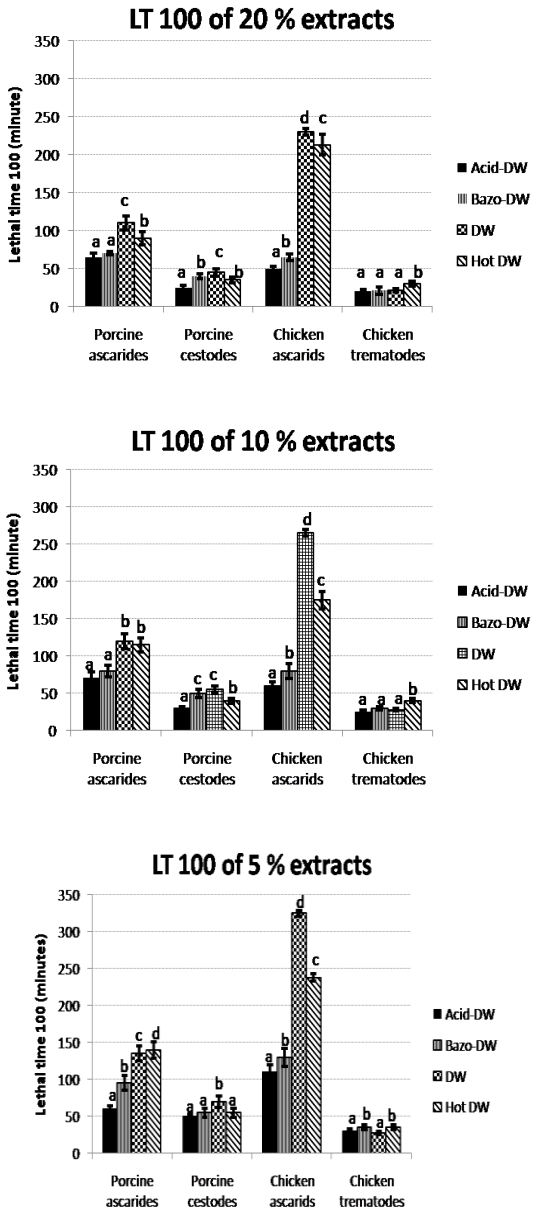


**Fig.1: The LT100 values of extracts at the concentration of 20 % (A), 10 % (B) and 5 % (C).**

Means values of extracts to each endoparasite with different superscripts (a, b, c, d, e) are significantly different ($p < 0.05$) by one-way ANOVA and *Post – hoc* Fisher's least significant difference test.

From Figure 1, we observed that acid-DW had the significantly lowest LT 100 with all of tested endoparasites in all of extracts at 20 % (Figure 1A), 10 % (Figure 1B) and 5 % (Figure 1C). Folowing acid-DW extracts, bazo-DW or hot-DW extracts hold the second high effect in 11 of 12 tests, and with only one exception in case of 5 % extracts with chicken trematodes, in which they processed the lowest efficacy with the longest LT100 (Figure 1C). DW extracts had the weakest effect with the longest LT100 in 11 of 12 tests. There was only one exception of 5 % extracts with chiken trematodes, in which this extract had the highest effect (Figure 1C).

In order to evaluate the sensitivity and resistance of different endoparasites with *Punica gramatum* L bark extracts, we compared the LT100 values of different endoparasites with each extract. The results were shown in Figure 2.

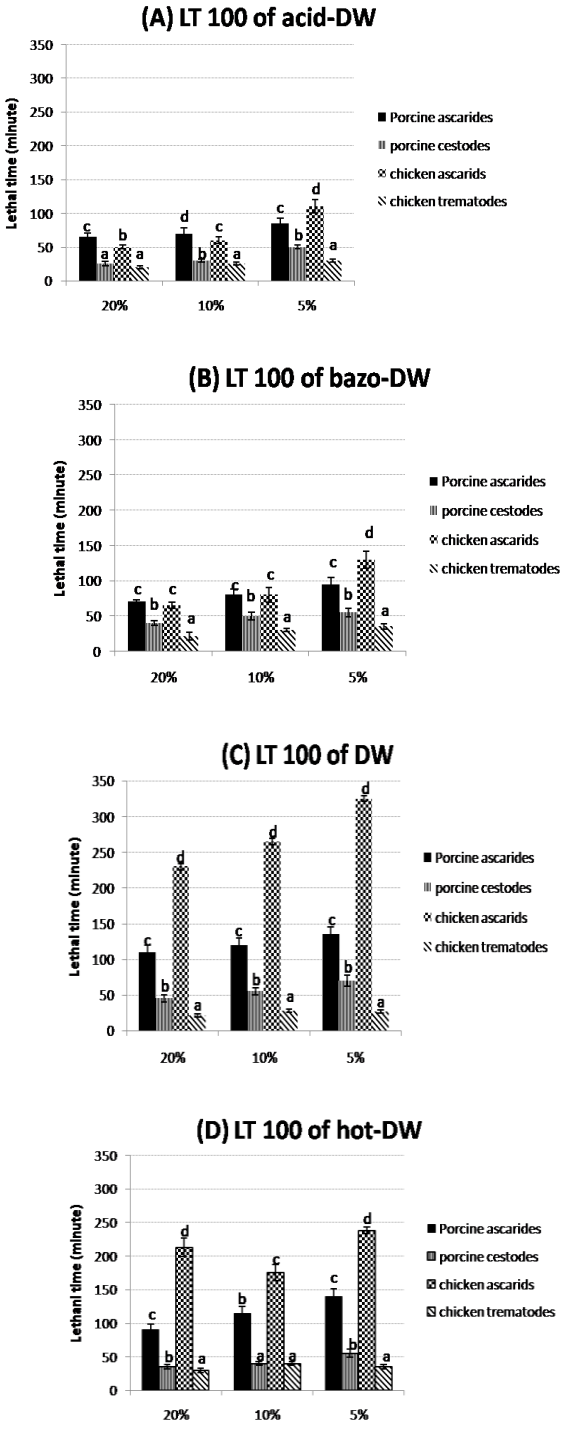


**Fig.2. The LT 100 values of 20 % acid-DW (Figure A), 20 % bazo-DW (Figure B), 20 % DW (Figure C) and 20 % hot-DW (Figure D).**

Means values of LT 100 at each concentration with different superscripts (a, b, c, d) are significantly different ($p < 0.05$) by one-way ANOVA and *Post – hoc* Fisher's least significant difference test.

From Figure 2, we observed that regardless of the different extracts and different concentrations, the chicken tremantodes was always the most sensitive parasite, which was shown by the significant lowest LT 100 values in all 12 cases (Figure 2A, 2B, 2C, 2D). Second to chicken tremantodes, porcine cestodes were also high sensitive with *Punica gramatum* L bark extracts. This parasite hold the

significant lowest LT100 in cases of 20 % of acid-DW extract (Figure 2A) and 20 % and 10 % of hot-DW extract (Figure 2D). For all of other 9 cases, this parasite possessed the second low LT 100 values (Figure 2A, 2B, 2C, 2D). Porcine ascarides and chicken ascarides showed high resistance against *Punica gramatum* L extracts, because they hold the first or second highest LT 100 to all 12 extracts. Porcine ascarids had significant highest LT 100 with 4 extracts, including 20 % acid-DW, 10 % acid-DW (Figure 2A), 20 % bazo-DW, 10 % bazo-DW (Figure 2B). Chicken trematodes had significant highest LT 100 with 10 of 12 cases, with only 2 exceptions in cases of 20 % acid-DW and 10 % acid-DW (Figure 2A).

## DISCUSSION

Our results proved the *in vitro* anthelminthic effect of *Punica gramatum* L bark extracts with endoparasites, including porcine ascarids, porcine cestodes, chicken ascarids and chicken trematodes. The extracts induced the death of these endoparasites with the dose-dependent manner. The potency of anthelminthic activity was inversely proportional to the lethal time taken for paralysis of the parasites, shown by the significant shorter lethal time in higher concentrations of the extracts. Our research is in accordance with the study of Darshan Shah *et al* [18], which reported the antihelmintic activity of *Punica gramatum* pulp. Because the difference in tested parasites and extraction methods, it is not able to compare the effect of the bark and the pulp. However, our study, together with the study of Darshan Shah *et al* [18], helps to confirm the traditional application of *Punica gramatum* plant, in which all parts of the plant can be used as traditional anthelmintics. At the concentration of 5 %, all tested extracts were able to paralyse 4 experiment endoparasites, suggesting the potential of applying this herb as the anti-parasite medicine plants. However, once a plant has proven its efficiency *in vitro*, further *in vivo* testing will be necessary to confirm the obtained results and evaluate risks, side-effects and future applicability [19].

Acid-DW showed the best effect to all of tested endoparasites. The different activity of the different solvents is due to their ability to dissolve the active anthelmintic compounds in the plants. Plants might consist of many anthelmintic substances which are related to the inhibition of cell division and to the formation and development of vital structures of parasites [20], or the inhibition of glucose uptake in the parasites and depletion of its glycogen synthesis, or activates nicotinic cholinergic receptor resulting in either persistent depolarization or hyperpolarisation [21]. The mechanisms by which acid was the favorable solvent to extract *Punica gramatum* L bark for the anthelmintic property was still not be able to explain on the basis of our present study, and the follow-up research is now in progress to identify the phytochemical compounds and also their soluability in different solvents . However, the study provided the preliminary information which suggestes that acid-DW was the most promissing and should be focused in the next step, including the clinical trial on infested animals and the investigation for the active compounds.

The results of this study showed the different in sensitivity and resistance of different endoparasites to the plant extract. The effect must be validated with *in vivo* testing on infested pigs and chicken to evaluate the real anthelminthic potential of this plant against porcine ascarids, porcine cestodes, chicken ascarids and chicken trematodes. There are many factors related to the physiology of the host that may alter the bioavailability of the active compounds. For instance, host pharmacokinetics may limit the amount of active compounds to reach to the parasites [8]. However, based on the different *in vitro* sensitivity of the endoparasites, we suggested that the different doses need to be used for different parasites in the *in vivo* test of the next step study. Within the concentrations that the drugs or plants/ plants preparation showed appropriate anthelmintic activity, the lower concentrations are always recommended in order to limit their side-effects on host animals.

## CONCLUSION

The present study shows that *in vitro* anthelmintic activity of *Punica gramatum* L bark extracts with animal endoparasites, and therefore partly explains the traditional application of this herb for parasite treatment. Extraction with $CH_3COOH$ and DW had the best efficacy in all of tested extracts and should be focused in the follow-up *in vivo* testing.

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