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Evaluation of pomegranate (*Punica granatum*) pericarp aqueous extract on *Eimeria* spp. from Japanese quails (*Coturnix japonica*)

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KEYWORDS

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Abstract Antibacterial, anti-inflammatory and antiparasitic properties have been associated with the extract of pomegranate (*Punica granatum*) in several animals and conditions. The Japanese quail (*Coturnix japonica*), originated from North Africa, Europe and Asia, is used worldwide as an experimental animal and model for aviculture. The current study investigated the effects of the pomegranate pericarp aqueous extract on the shedding, viability and morphometry of three *Eimeria* spp. from Japanese quails, besides the weight gain and genotoxic activity. Although the pomegranate is recognized by multiple properties, including anti-coccidial, in the current study the results are contrary. The treated group shed greater amount of oocysts; the sporulation times and viability were similar in both groups; despite some morphometric differences, these were not expressive; weight gains were similar; and the pomegranate had insignificant effect genotoxic. Finally, these results suggest that the pomegranate pericarp extract did not influence on *Eimeira* spp. from Japanese quails; therefore, the pomegranate pericarp extract is not suggested in the prevention/treatment of coccidiosis in Japanese quails, or at least not using methods of preparation and administration applied in this study.

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1. Introduction

Parasitoses represent a limiting factor in the breeding of poultry species, both in high-production commercial rearing farms or even in rustic breeding systems, where economic losses can be significant. Among the most pathogenic parasites, coccidiosis caused by *Eimeria* spp. is distinguished by severe enteropathy, which promotes anorexia, decreased reproduction and posture in adults, and is responsible for high levels of mortality in young animals [1–5].

Poultry coccidiosis is mainly controlled by the use of chemotherapeutic agents. However, several studies have targeted the use of herbal medicines, which reduces costs and must be effective in the prevention and/or treatment of coccidiosis. Studies have reported anti-inflammatory, antioxidant, antibacterial and antiparasitic (including anti-coccidial) properties associated with the extract of pomegranate (*Punica granatum*) in several animals, parasites and conditions [6–12].

Japanese quails (*Coturnix japonica*) has excelled in aviculture, due to increased consumption of exotic meats and eggs, and represents an alternative to chicken production. Furthermore, in recent decades, it has become an important experimental animal for scientific research, due to short life cycle and greater resistance to many poultry diseases [13–19].

Berto et al. [20] characterizes *Eimeria bateri*, *Eimeria tsunodai* and *Eimeria uzura*, which are commonly encountered on farms breeding Japanese quails provisioning an algorithm designed to enable reliable identification during routine diagnosis and experimental studies.

In this context, the present study investigated the effects of the aqueous extract of pomegranate pericarp on the shedding, viability and morphometry of three *Eimeria* spp. from Japanese quails, besides the weight gain and genotoxic activity.

2. Material and methods

2.1. Experimental Japanese quails and treatments

Eight one-day-old Japanese quails were obtained from a commercial rearing farm located in the Municipality of Seropédica in the State of Rio de Janeiro, Brazil. The chicks were transported to the Universidade Federal Rural do Rio de Janeiro (UFRRJ), and were reared and fed a single cage without anticoccidial additives. Feed and water were administered *ad libitum*. Chicks were randomly assigned to two experimental groups consisting of 8 individuals per group. Group 1 was fed with aqueous extract of pomegranate pericarp and were challenged (treated); Group 2 received an untreated standard diet and were challenged (control). Sample processing and data analysis were conducted at the Laboratório de Coccídios e Coccidioses and in the Departamento de Biologia Animal (Biology area), both located at UFRRJ. The experiments were approved by Ethics Committee in Research of the UFRRJ.

2.2. Preparation of the pomegranate pericarp extract

Pomegranate pericarps were obtained from fruit collected from trees in the municipality of Seropédica, state of Rio de Janeiro, Brazil. The samples were authenticated by Herbaria Technician Thiago Azevedo Amorim (Departamento de Botânica, Instituto de Biologia, UFRRJ, Brazil) and the exsiccate was placed in the herbaria under protocol RBR No. 35804. Pomegranate pericarp aqueous extract was prepared according to the method described by Amorim and Borba [21] with some modification. Pericarps were cold-dried under ambient conditions, pulverized and dissolved (5 g) under boiled (90 °C/194°F) distilled water (100 ml). This pomegranate aqueous extract were supplied for drinking water from 5 to 11 days of age.

2.3. Eimeria spp. challenge

The adult Japanese quails from the commercial rearing farm used to provide the chicks were naturally parasitized by *E. bateri*, *E. tsunodai* and *E. uzura*. An inoculum for experimental infection was produced by recovering and isolating oocysts from fecal samples of positive Japanese quails by flotation in Sheather's sugar solution (S.G. 1.20) according to the protocol of Duszynski and Wilber [22]. The oocysts were preserved in a 2.5% (w/v) solution of $K_2Cr_2O_7$ to induce sporulation and maintained in a refrigerator (2–5 °C) until use. *C. japonica* chicks were inoculated at the age of 7 days, using inocula, quantified using a Neubauer chamber, containing approximately 6.5×10^4 sporulated oocysts per chick, at the approximate proportion of: 50% *E. bateri*; 25% *E. tsunodai*; and 25% *E. uzura*.

2.4. Assessment of the shedding, viability and morphometry of the Eimeria spp. oocysts

The number of oocysts shed in the feces was determined on days 2, 3, 4, 5, 7, 9, 11, 14, 16, 18, 21, 23, 25 and 28 after infection (DAI). Enumeration of oocysts per gram of faeces (OoPG) was performed according to Menezes and Lopes [23], as modified by Cardozo et al. [18]. The viability of the oocysts was evaluated from the shedding of the oocysts until 120 h after, maintaining the fecal sample in a thin layer (~5 mm) of $K_2Cr_2O_7$ 2.5% solution in Petri plates, and incubated at 23–28 °C. During this period was observed daily the proportion of oocysts non-sporulated, with sporoblasts and sporulated within of the same sample. *Eimeria* spp. oocysts from Japanese quails were identified using the characteristics and the algorithm designed by Berto et al. [20]. Morphological measurements, given in micrometres, were made using a Carl Zeiss binocular microscope with an apochromatic oil immersion objective lens and an ocular micrometer (K-15X PZO) Poland. Size ranges are shown in parentheses followed by average and shape index (L/W ratio).

2.5. Assessment of the genotoxic activity

For estimating genotoxic property, a Japanese quail from each group, chosen randomly, were used to collect blood samples on days 2, 4 and 28 post infection. Smears slides were prepared and all were code, fixed with methanol and stained with Giemsa solution. For micronucleus (MN) presence, two thousand erythrocytes from each animal (one thousand/slide) were scored and the total of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs) was determinated.

2.6. Statistical analysis

Three statistical methods were employed for comparison between groups: (1) Student's *t*-test was used to compare measurements of the length, width and shape-index of the oocysts and sporocysts of individual *Eimeria* spp., besides, compare weight gain. The software Microsoft® Excel 2007 was used to calculate the mean, variance, degree of freedom and *p* value [24–26]; (2) Linear regression to determine the distribution of *Eimeria* spp. sporulated oocysts using methods proposed by Norton and Joyner [27] and subsequently modified by Sampaio [25] and Berto et al. [26]. The graphs and coefficient of regression line were obtained using the software Microsoft Excel 2007®; (3) χ^2 test at significant level $p < 0.05$ to compare the genotoxic assay results.

3. Results

At 28 DAI, the Japanese quails of the experimental groups were apparently healthy. The means of weight gain were not significantly different ($P > 0.05$). The mean weights were 144.7 g (125.6–163.6) in the treated group and 132.6 g (117.0–149.9) in the control group.

3.1. Oocysts shedding, viability and morphometry

The treated group shed a total of 1,361,310 oocysts, being 920,924 (68%) *E. bateri*, 202,605 (15%) *E. tsunodai* and 237,781 (17%) *E. uzura*; whereas, the control group shed a total of 1,195,608 oocysts, being 849,425 (71%) *E. bateri*, 268,465 (22%) *E. tsunodai* and 77,718 (7%) *E. uzura*. Graphs relating the results of OoPG, by groups, *Eimeria* spp. and

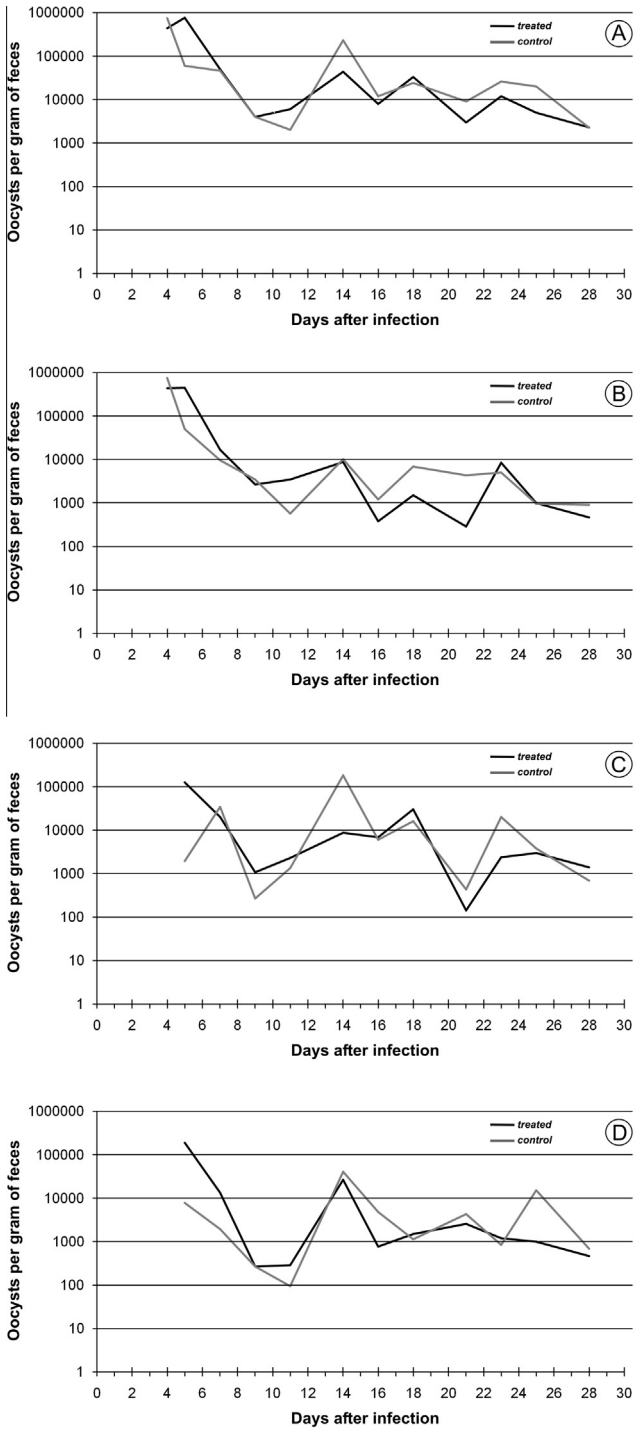

Figure 1 Dynamics of oocyst shedding of oocysts of *Eimeria* spp. recovered from Japanese quails (*Coturnix japonica*) treated and untreated (control) with pomegranate pericarp extract: (A) *Eimeria* spp.; (B) *Eimeria bateri*; (C) *Eimeria tsunodai*; and (D) *Eimeria uzura*.

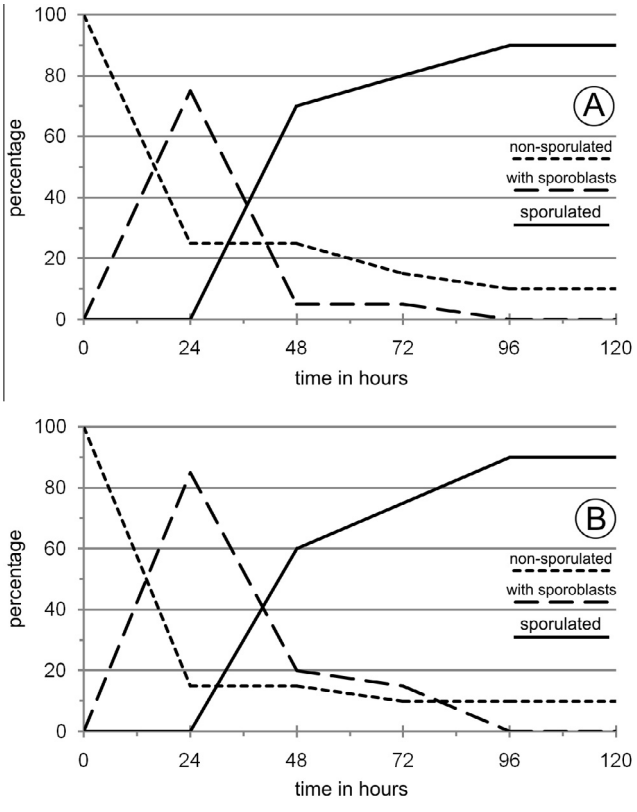

Figure 2 Sporulation time and viability of the oocysts of *Eimeria* spp. recovered from Japanese quails (*Coturnix japonica*) treated and untreated (control) with pomegranate pericarp extract: (A) treated; (B) control.

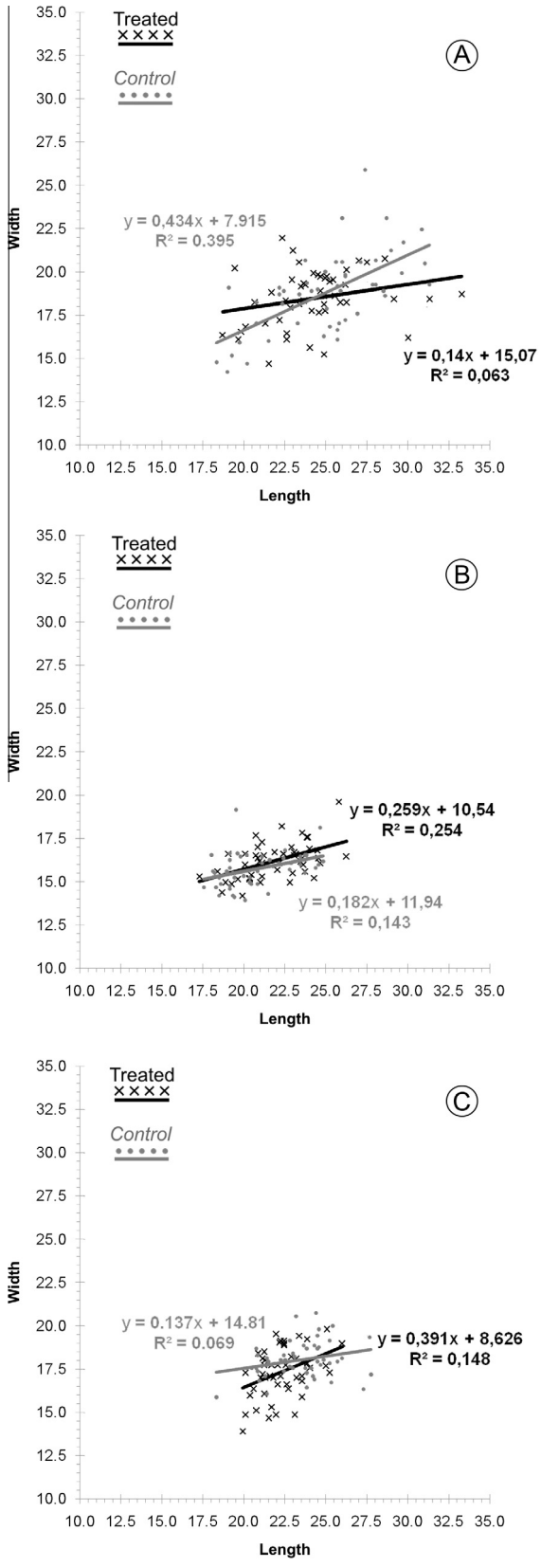

Figure 3 Comparative linear regressions of the sporulated oocysts of *Eimeria* spp. recovered from Japanese quails (*Coturnix japonica*) treated and untreated (control) with pomegranate pericarp extract: (A) *Eimeria bateri*; (B) *Eimeria tsunodai*; and (C) *Eimeria uzura*.

DAI of shedding, can be observed in Fig. 1. After shedding, the oocysts were non-sporulated, but 70% sporulated by day three, in the two experimental groups (Fig. 2). The three *Eimeria* spp. in both groups were polymorphic, as verified by linear regression analysis, which demonstrated multiple variations for values of width on length and low value of R^2 (Fig. 3). Table 1 shows the means comparisons between groups in all parameters of oocysts and sporocysts, where is observed that *E. tsunodai* and *E. uzura* had length and width of the oocysts significantly different.

3.2. Genotoxic activity

The numbers of micronucleated erythrocytes were equivalent in both experimental groups, indicating that the aqueous extract of pomegranate pericarp had no mutagenic potential (Table 2).

4. Discussion

Primarily, this study aimed to evaluate the effect of pomegranate on parameters of the oocysts of *Eimeria* spp. In this sense, although some data have been collected from hosts such as weight gain and genotoxic activity, other data as diarrhea, macro- and microscopic lesions, etc. were suppressed.

Pomegranate is widely recognized by multiple properties, including anti-coccidial. Among numerous studies, recently Wang et al. [28], Al-Mathal and Alsalem [29] and Dkhil [12] reported the efficiency of the pomegranate extract in the treatment of coccidiosis in mice and rabbits. However, in the current study, the results are contrary: (1) the treated group shed greater amount of oocysts, (2) the sporulation times were similar in both groups, and therefore, were viable and infective similarly, (3) despite some morphometric differences observed in *E. tsunodai* and *E. uzura*, these were not expressive, (4) weight gains were similar and, finally, (5) the pomegranate had insignificant effect genotoxic.

There may be some assumptions for these divergences, as the different methods of preparation and administration of pomegranate extract. Some authors dissolve the pomegranate powder in cold water [29]; extract pomegranate powder by percolation with methanol [12]; use other parts of the pomegranate tree, such as root and stem [9]; administer by gastric tubes [29]; etc. Furthermore, the efficiency was clearly demonstrated in coccidiosis of mice and rabbits, and, therefore, may be different in poultry.

The polymorphism of these three *Eimeria* spp. had been previously characterized by Berto et al. [20]; however, in this study the linear regressions demonstrated tendencies and similarities of each species between experimental groups. It may be observed that, despite of the polymorphism, the regression lines and points remained close or superimposed, revealing similar measures and tendencies (Fig. 3). The Student's t-test resulted in significant differences in the length and width of the oocysts of *E. tsunodai* and *E. uzura*. This result is similar to studies of Gomez et al. [24] and Berto et al. [30] conducted with coccidia from birds conditioned in distinct sites. These studies and Parker and Duszynski [31] concluded that immunity is one of the factors associated with the differences in the morphometry. In this way, as pomegranate has anti-inflammatory potential widely recognized, it is possible that

Table 1 Means comparisons of *Eimeria* spp. oocysts recovered from Japanese quails (*Coturnix japonica*) treated and untreated (control) with pomegranate pericarp extract.

Means			Oocyst samples (μm)	
			Treated (n = 50)	*Control (n = 50)*
Eimeria bateri	Oo[A]	Length	25.1 (18–31)[a]	24.3 (19–33)[a]
		Width	18.5 (15–22)[a]	18.8 (14–26)[a]
		Shape index	1.3 (1.0–1.9)[a]	1.3 (1.0–1.6)[a]
	Sp[B]	Length	11.2 (10–12)[a]	11.5 (10–13)[a]
		Width	7.1 (6–8)[a]	7.4 (6–8)[a]
		Shape index	1.6 (1.5–1.8)[a]	1.6 (1.4–1.6)[a]
Eimeria tsunodai	Oo	Length	21.9 (17–26)[a]	20.8 (18–25)[b]
		Width	16.2 (14–20)[a]	15.7 (14–19)[b]
		Shape index	1.4 (1.1–1.6)[a]	1.3 (1.0–1.5)[a]
	Sp	Length	11.1 (9–13)[a]	10.8 (10–12)[a]
		Width	5.9 (5–7)[a]	5.7 (5–6)[a]
		Shape index	1.9 (1.6–2.1)[a]	1.9 (1.7–2.2)[a]
Eimeria uzura	Oo	Length	22.3 (20–26)[a]	23.3 (18–28)[b]
		Width	17.4 (14–20)[a]	18.0 (16–21)[b]
		Shape index	1.3 (1.1–1.6)[a]	1.3 (1.1–1.7)[a]
	Sp	Length	13.0 (12–14)[a]	13.6 (13–14)[b]
		Width	6.2 (6–7)[a]	6.4 (5–7)[a]
		Shape index	2.1 (1.9–2.3)[a]	2.1 (2.0–2.4)[a]

Different letters in each line denote statistically significant differences ($p < 0.05$) by the Student's *t*-test.
[A] Oocyst values.
[B] Sporocyst values.

Table 2 Number of micronucleated erythrocytes from Japanese quails (*Coturnix japonica*) treated and untreated (control) with pomegranate pericarp extract.

Groups	Number of analyzed cells	Number of micronucleated erythrocytes		
		2 DAI[A]	4 DAI[A]	28 DAI[A]
Treated	2000	0	1	0
Control	2000	0	1	1

[A] DAI: days after infection.

this anti-inflammatory effect has induced these morphometric differences between experimental groups.

Finally, these results suggest that the pomegranate pericarp extract did not influence on weight gain of Japanese quails, in the shedding of oocysts of *Eimeira* spp., in the viability and in the genotoxic activity, having only influence on the morphometry of the oocysts of *E. tsunodai* and *E. uzura*. Thus, the pomegranate pericarp aqueous extract is not suggested in the prevention/ treatment of coccidiosis in Japanese quails, or at least not using methods of preparation and administration applied in this work.

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