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Efecto anticoccidial de los extractos de taninos de *Tara spinosa* (Molina) Britton & Rose sobre la supervivencia de los ovocitos apicomplejos de *Eimeria* sp.

Anticoccidial effect of *Tara spinosa* (Molina) Britton & Rose tannin extracts on survival of the apicomplexan *Eimeria* sp. Oocytes

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RESUMEN

Se estudió un extracto acuoso de taninos hidrolizables del pericarpio de plantas de tara utilizando frutos de vainas recolectadas en las tierras altas de la región La Libertad en el norte del Perú. La presencia de taninos en el extracto se verificó mediante el uso de la prueba de tricloruro de hierro ($FeCl_3$) y se calculó una concentración de 45% de taninos análogos al ácido gálico utilizando un método espectrofotométrico. El extracto se aplicó sobre ooquistes vivos de *Eimeria* sp. presentes en la vacuna viva EVANT® (esta formulación contiene especies de coccidios con patogenicidad específica para aves de corral). Los ooquistes fueron expuestos a diez tratamientos con concentraciones aumentadas y dos controles con cinco réplicas para cada uno. La inspección microscópica se realizó regularmente después del tiempo de exposición con el objetivo de detectar la integridad de la membrana celular. Los resultados muestran que el mayor número de ooquistes afectados por los extractos se produjo a una concentración de 50000 ppm después de 48 horas de exposición *in vitro,* por lo que sugiere que el extracto de tanino de tara tiene propiedades anticoccidiales sobre los parásitos que afectan a las aves de corral. Se recomiendan estudios adicionales para investigar el efecto de los extractos de tara sobre los ovocitos *in vivo,* y su eventual incorporación como ingrediente en alimentos para aves de producción avícola.

Palabras clave: *Eimeria*; ooquistes; aves de corral; taninos; coccidios.

ABSTRACT

An aqueous extract of hydrolysable tannins from the pericarp of tara plants was studied using pod fruits collected from the highlands of La Libertad region in northern Perú. The presence of tannins in the extract was verified through the use of the iron trichloride ($FeCl_3$) test and a concentration of 45% of tannins analogous to gallic acid was calculated using a spectrophotometric method. The extract was applied on live oocysts of *Eimeria* sp. present in the EVANT® live vaccine (this formulation contains coccidian species with specific pathogenicity to poultry birds). The oocysts were exposed to ten treatments with increased concentrations and two controls with five replicates for each one. Microscopic inspection was carried out regularly after the time of exposure aiming to detect cell membrane integrity. The results show that the greatest number of oocysts affected by the extracts occurred at a concentration of 50000 ppm after 48 hours of *in vitro* exposure, so it suggests that the tannin extract from tara has anticoccidial properties on parasites affecting poultry. Further studies are recommended to investigate the effect of tara extracts on oocytes *in vivo*, and its eventual incorporation as ingredient in bird feed for poultry production.

Keywords: *Eimeria*; oocysts; poultry; tannins; coccidia.

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INTRODUCTION

Coccidiosis is a parasitic disease affecting the gastrointestinal tract of various animals (Razavi et al., 2024; Gurbanova, 2025). The causative agent is the apicomplexan protozoon *Eimeria* (López-Osorio et al., 2020), which leads to significant economic losses in the poultry industry (Mares et al., 2023). Various control strategies are used, including the biological control using predatory fungi (Lozano et al., 2024), anticoccidial drugs (Sánchez et al., 2024), vaccines and natural products (Cheng et al., 2022). Nevertheless, resistance has been observed to some pharmacological treatments (Cheng et al., 2022).

Agents that possess anticoccidial activity, particularly from plant sources, as has been found in the peel of the pomegranate (*Punica granatum*) fruit (Ahad et al., 2018), grapevine (*Vitis vinifera*) seeds (Abbas et al., 2020), extracts of garlic, onion (*Allium* sp.), and moringa trees (*Moringa oleifera*) leaves (Abo-Aziza et al., 2022), among others. Studies on secondary metabolites existing in plant parts such as polyphenols, saponins and certain organic acids have been implicated in controlling oocysts of numerous *Eimeria* species (e.g., *E. acervulina, E. mivati, E. maxima, E. tenella, E. ecatrix, E. praecox, E. morena, and E. hagani*). (Abudabos., 2022). Likewise, sugarcane bagasse polysaccharides show a modulating effect on immune responses in broiler chickens that might protect against coccidian infections (Awais et al., 2018).

Tara spinosa (Molina) Britton & Rose is a native leguminous plant from Perú known by the name of tara (Sangay-Tucto et al., 2024), whose leaves are extensively used in popular medicine to prepare infusions to fight parasites and fleas (Raimondi, 1857). Recent studies reveal that its antimicrobial action is due to the presence of tannins, flavonoids steroids and alkaloids. Other studies found that concentrations of hydroalcoholic extract of tara pods did increase activity against β-hemolytic streptococci (De La Cruz-Noriega et al., 2023).

Melanoma therapies based on nanoencapsulated standardized extracts of *Caesalpinia spinosa* have also displayed antitumor activity (Lasso et al., 2020). In breast cancer, it significantly delays tumor development and the frequency of macrometastases due to pathways related to increased intracellular calcium ions , and activation of autophagy (Brotons-Canto et al., 2023). Tara extract also inhibits the replication of murine hepatitis virus (MHV)-A59 by attenuating reactive oxygen species, unfolded protein responses, and autophagy (Prieto et al., 2023). Tannins also present antioxidant activity that diminishes the harmful effects of reactive oxygen species (ROS) produced during the freezing and thawing process in the cryopreservation of animal spermatozoa (Liman et al., 2022).

The purpose of this study was to investigate the effect of aqueous extracts of hydrolysable tannins from tara pods on the survival of *Eimeria* oocysts *in vitro*. The results highlight the capability of tara extracts to control this avian parasite and its potential use in poultry production.

METHODOLOGY

The ethical standards of the Universidad Privada Antenor Orrego and the Universidad Nacional de Trujillo were followed, aligned with the International Research Standards (Directive 2010/63/EU, 2010). The study was carried out during pandemic times. Annex 28 of the sworn declaration of responsibility for the quality of the study in good ethical research practices of the Manual of Procedures of the Research Ethics Committee of the Universidad Nacional de Trujillo was signed on March 31, 2022.

Plant source and taxonomy

Tara plant fruits were collected in La Cuesta, district of the Otuzco Province, La Libertad Region (altitude 2100m, latitude 7°54′31″S, longitude 78°41′06″W). Taxonomic identification as *Tara spinosa* (Molina) Britton & Rose (Fabaceae) was made in the Antenor Orrego Herbarium (HAO) (certificate Nº16-2019-HAO-UPAO). This plant belongs to the family Fabaceae, subfamily Caesalpinioideae, and tribe Caesalpinieae (WFO, 2023).

Laboratory facilities

The preparation of the extract, the qualitative and quantitative identification of tannins as well as the pilot exposure of *Eimeria* sp. were carried out in the Laboratorio de Investigación en Fisiología y Fisioapatología del Metabolismo de Alimentos en la Ruta de Investigación Nutricional (MARINUTRI). Facultad de Farmacia y Bioquímica, Universidad Nacional de Trujillo. Perú. The incubation and assessment of the oocysts were carried out in the laboratories of the Facultad de Medicina Veterinaria y Zootecnia. Universidad Privada Antenor Orrego.

Tannin extraction

Aqueous extraction of tannins from the tara pericarp was obtained as follows: A mass of 200 g was weighed from fully developed fruit pods without defects, with red to light brown tones; the seeds were separated, and the pericarp was threshed, ground, and sieved to obtain granules of 0.5 mm in diameter; it was then dehydrated in a oven at 40 °C until dehydrated powder was obtained. A 0.4 g fraction was weighed, and subsequently dissolved in 70 mL of distilled water, heated at 60 °C for 10 minutes. It was left to cool and the extract was obtained by vacuum filtered with Whatman No. 40 paper using distilled water; the result of the extraction was kept in a covered container of amber glass protected from light with aluminum foil (Miranda-Martínez, 2012).

Chemical determination of tannins

Qualitative determination. The method of Lock

(Lock, 2016) used the ferric chloride ($FeCl_3$) reaction to determine the presence of tannins.
Quantitative determination. The Folin-Ciocalteu reagent (García et al., 2015) was used to determine total polyphenol content by observing a blue coloration formed and quantified using a spectrophotometer (Thermo Spectronic Genesys 10 UV scanning, Thermo Scientific, USA) at a wavelength of 760 nm. (León & Mancheno, 2020). A calibration curve was obtained by plotting the absorbance on tannin concentration.

Treatments
Dilutions were prepared to achieve the following final concentrations, each becoming a treatment, namely: T0- (Negative control), T0+ (Positive control), T1 (1000 ppm), T2 (2000 ppm), T3 (3000 ppm), T4 (5000 ppm), T5 (10000 ppm), T6 (20000 ppm), T7 (30000 ppm), T8 (50000 ppm), T9 (100000 ppm) and T10 (140000 ppm).

Preparation of oocysts of *Eimeria* sp.
A 1000-dose vial of live vaccine EVANT® (EMA, 2018) was purchased. According to the manufacturer (Laboratorios HIPRA Spain), a volume of 0.007 mL of undiluted EVANT® vaccine contains a blend of five *Eimeria* strains whose number of oocysts in the final vaccine formulation was determined by the manufacturer using *in vitro* procedures at the time of blending. In the live vaccine, the active substances and their approximate number of sporulated oocysts derived from precocious attenuated lines of coccidia were: *Eimeria acervuline* strain 03 (332-450), *Eimeria maxima* strain 013 (196-265), *Eimeria mitis* strain 006 (293- 397), *Eimeria praecox* strain 007 (293-397), and *Eimeria tenella* strain 004 (276-374). The vaccine had the oocysts suspended in HIPRAMUNE T solvent (which contains Montanide IMS as an adjuvant, and the excipients Brilliant Blue E133, Red AV E129 and Vanillin).

Measurements
Different concentrations of tannin extracts from tara were applied to oocysts of *Eimeria* sp. and a microscopic examination of the structural integrity of its outer membrane was inspected using a light compound microscope. A Neubauer hemocytometer was used for cellular measurements.
The concentration of tannins was set in equivalence to its gallic acid concentration. The concentration of a main solution at 150000 ppm was prepared, from 10 g of dehydrated powder of which 5 g was taken. It was dissolved with 15 mL of distilled water, heated at 60 °C for 10 minutes, cooled, and brought up to 15 mL It was vacuum filtered with Whatman No. 40 paper. This concentrated solution was stored in an amber glass bottle protected with aluminum foil. From it, different treatment dilutions were prepared (Malada et al., 2022).

Treatment conditioning as a challenge to the oocysts of *Eimeria* sp.
Aliquots of the 1X solution of tara extract in various concentrations (see Table 1) were added to 60 Eppendorf tubes. Distilled water was used as a negative control. A 5% formaldehyde aqueous solution was used as positive control. A volume of 50 µL of EVANT® vaccine obtained at room temperature was added to each tube. This volume contained collectively between 7957 to 10779 oocysts. After the application of the tannin treatment, 20 µL of each tube was placed in a Neubauer hemocytometer to be visualized at 400X in an optical microscope in five repetitions. Observations on the effect of the tannin extract on the oocysts membranes were taken at 0, 12, 24, 48 and 72 hours (Fatemi et al., 2015). The duration times of exposure were selected given that the longer durations would confound natural degradation from the experimental treatment effects.
Chemical assessment based on the ferric chloride showed that the tara extracts possessed hydrolyzable polyphenols (e.g., tannins). Microscopic inspection of the oocysts challenged by tannin extracts for each treatment are presented in selected photomicrographs (Figure 1). The negative control (T0-), as expected, did not show alterations in the cell membrane. Treatments T1 to T7 had varying degrees of cell disruption. More clearly, treatment T8 (50 000 ppm tannin) displayed an irreversible disruption of cell membrane integrity.

Statistical analysis
The statistical analysis was conducted using a Completely Randomized Design (CRD), with 5 replicates per treatment. The main response variable was the structural damage to oocysts (e.g., degranulation of its membrane).

Table 1
Preparation of dosage treatments (Volume, µL)

Treatment	Tannin (ppm)	EVANT*	Formaldehyde	Tannin 150000 ppm	Distilled water
T0+	0	50	140	0	810
T0-	0	50	0	0	950
T1	1000	50	0	7	943
T2	2000	50	0	13	937
T3	3000	50	0	20	930
T4	5000	50	0	33	917
T5	10000	50	0	67	883
T6	20000	50	0	133	817
T7	30000	50	0	200	750
T8	50000	50	0	333	617
T9	100000	50	0	667	283
T10	140000	50	0	935	15

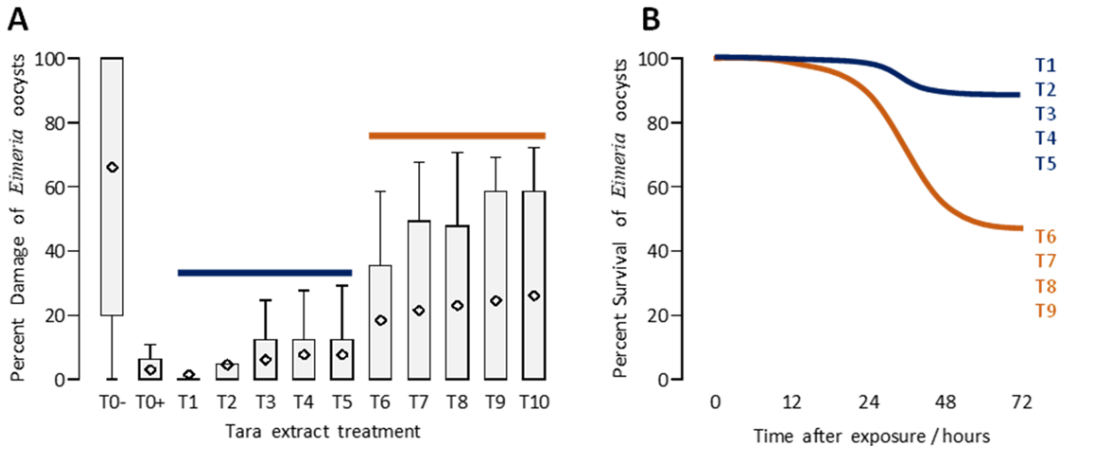

Figure 1. (A) Percent damage of *Eimeria oocysts* under increasing concentration of tara extract. Bars above the treatment results reflect the Duncan Multiple Comparison tests. (B) Survival trend of *Eimeria oocysts* at distinct evaluation times. Lines indicate the overall tendency of the results obtained experimentally, and summarize the treatments indicated. Blue line indicates a similar trend between treatments T1 to T5. Red line indicates a similar trend between treatments T6 to T10.

This design was chosen to compare the effect of different tannin extract concentrations (treatments) on the structural integrity of the oocyst cell. For this purpose, a linear model of analysis of variance (ANOVA) was applied, given by the linear model $Y_{ij} = \mu + T_i + E_{ij}$; where μ is the overall mean effect, T_i is the effect of the i^{th} treatment (i.e., independent variable, namely, the extract concentration of tannins), and E_{ij} the experimental error of the ij^{th} experimental unit Y_{ij}. The averaged results were evaluated using the Tukey's test ($p < 0.05$). All statistical analyses were performed using SPSS v.24 software (IBM Corp., 2020).

RESULTS AND DISCUSSION

The effect of the tara extract on the cell integrity of *Eimeria* coccidia is presented in Figure 1A. It shows that the increase in concentration relates directly with cell damage. The Duncan multiple comparison test (significance level α = 0.05) separated the treatments into two distinct groups, whose members had no statistically significant differences among them show the percentage survival of the *Eimeria* sp. oocysts for each diluted extract at a given exposure duration. It is observed that the highest effect of the extract equivalent to 50000 ppm occurred after a 48-hour exposure and that this effect did not increase in a statistically significant manner beyond this concentration and duration. Figure 1B displays the general tendency of survival of coccidia under these treatments at distinct duration of exposure. It shows the survival rate of *Eimeria* oocysts after exposure to the various tannin extract treatments in various exposure times. Some patterns can be discerned from these results. First, the damage that occurs to the oocysts is greater with the length of exposure, as well as the damage is concomitant with the concentration of tannins in the prepared extracts.

Two for each treatment. (LM magnification 400X; bar 50 µm). Different species of *Eimeria* are observed: *E. acervulina*, the oocysts are ovoid with dimensions of 17.7 – 22.2 x 13.7 – 16.3 µm, the oocyst wall is smooth, thinner at the narrow pole and with a barely noticeable micropyle. *E. maxima*, the oocysts are ovoid and larger than in the rest of the species, with dimensions in a range of 21.4–42.5x 16.5 – 29.8 µm, the oocyst wall is slightly yellowish, rough, and lacking a micropyle. *E. tenella* the oocysts are ovoid with dimensions in a range of 14.2 – 31.2 x 9.5 – 24.8 µm, the oocyst wall is smooth and lacks a micropyle. *E. mitis*, the oocysts are ovoid with dimensions in a range of 12.2 – 15.3 x 9.5 – 14.8 µm. *E. praecox*, the oocysts are ovoid with dimensions in a range of 16.3 – 18.7 x 11.7 – 16.3 µm.

The aim of this study was to investigate the effect of hydrolysable tannins obtained from the pericarp of the tara fruit on oocyst membrane integrity of the apicomplexan parasite *Eimeria* sp. Previous studies have attributed this capacity to control the parasite to the presence of tannins found in distinct plant parts (Lock, 2016). The concentration of tannins in tara plants has been found to vary depending upon the geographical provenance, nutrition and plant organ (Richane et al., 2022). The values obtained in this study are similar to what was been found in previous studies of the neighboring localities of the Andean zone of Perú (Valdiviezo-Campos et al., 2024). Microenvironmental conditions among them could have a significant effect on the production of tannins by tara plants (Murga-Orrillo et al., 2023; Chambi et al., 2013).

Lesions and disintegration of the external membranes were observed in Figure 2, with levels of damage ranging from degranulation and deformation of the membranes to disintegration of the oocyst. Probably, this could be the result of enzymatic inhibition on the membranes of microorganisms as well as the deprivation of substrates necessary for their metabolism (Engels et al., 2011). Plasma membrane integrity is intrinsically associated to resistance and repair capacity of the organism, both of which are influenced by host genetics and environmental factors. Imbalances compromise the survival of the oocyst (Ammendolia et al., 2021).

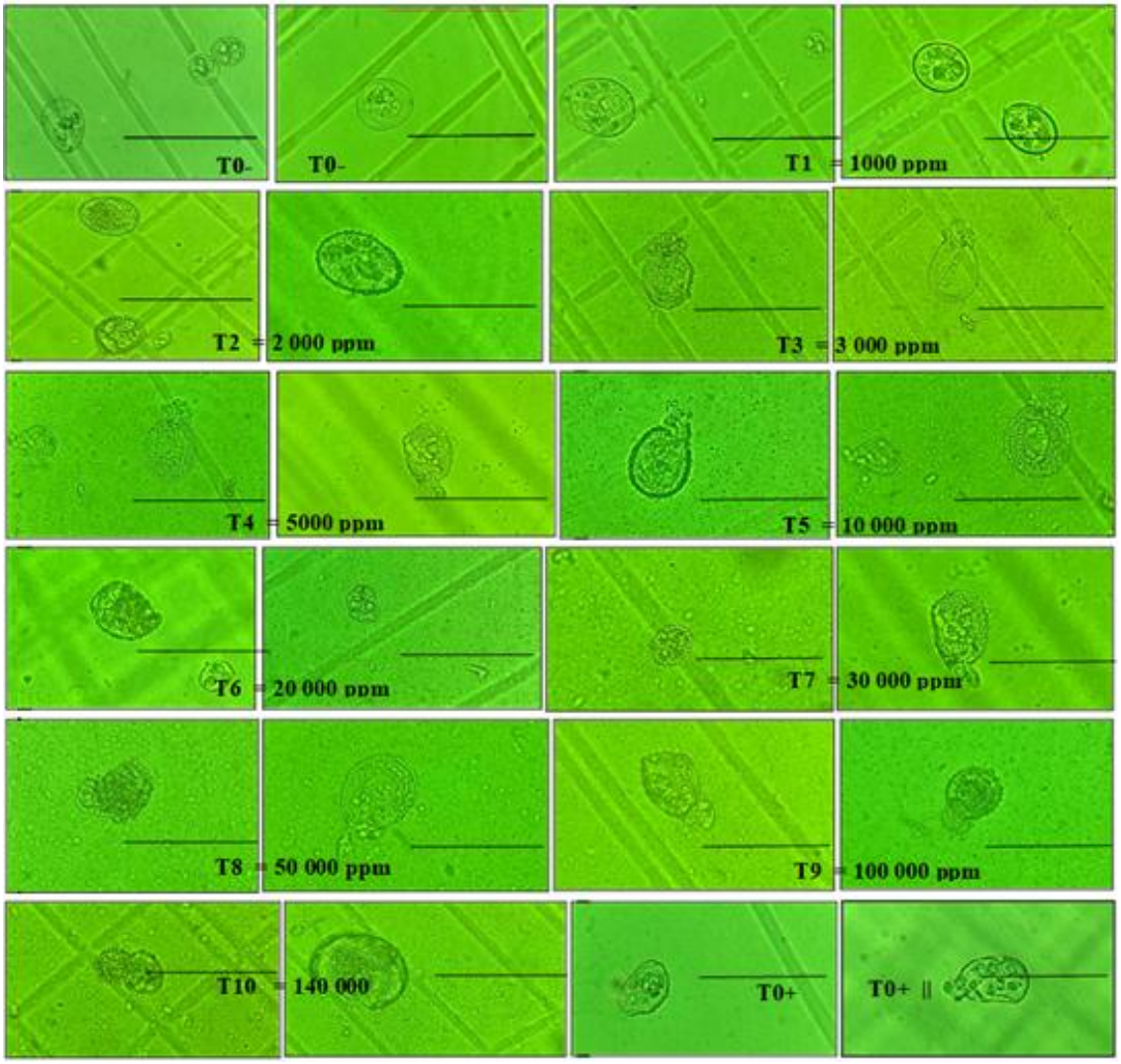

Figure 2. Photomicrographs of *Eimeria oocysts* exposed to different tannin treatments, two for each treatment (LM magnification 400X; bar 50 µm).

Hydrolysable tannins are known to interact with the antihelmintic benzimidazole and that the interaction becomes stronger by increasing the number of free galloyl groups and the molecular flexibility of the tannins (Sillanpää et al., 2023). Likewise, as a defense mechanism, plants might accumulate hydrolysable gallotannins such as ellagitannin, the 1-0-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose, whose accumulation in strawberry plants occurs as a response to its interaction with the anthracnose-producing fungal pathogen *Colletotrichum fragariae*. (Mamaní et al., 2012).

Tannins have garnered a great deal of attention as an alternative for growth promoters applied in poultry feed because of their antibiotic, antioxidant and anti-inflammatory properties (Choi & Kim, 2020) (Lee et al., 2021). Tests evaluating chestnut tannin additives in broiler chicken feed have found an alteration of the metabolic cecum phenotype of chickens which suggests the importance of improving the intestinal health of broiler chickens (Zea y col, 2019). Other reports also found that broiler chicken fed with a basal diet supplemented with 0.1% of tara gum showed a positive response in live weight, weight gain and intestinal morphology. These parameters suggest an enhancement of beneficial bacterial prevalence at the expense of pathogenic ones. Beta-galactomannans present in tannins haven been shown to adhere to *Salmonella fimbriae*, leading to their decrease in abundance and eventual eradication (Oyofo et al., 1989).

Tannins also promotes protein denaturation and its precipitation in pathogenic microorganisms (Olivas-Aguirre et al., 2015). Tannins are especially prone to have a dissuasive effect in insects' guts, forming toxic compounds to the insect (Barbehenn & Constabel, 2011). These mechanisms may point out to the possible mechanism of action of tannins on the cellular physiology of *Eimeria* oocysts (Güven et al., 2013). However, in insects, it is suggested that their ability to tolerate tannins ingested from plant leaves are modulated in their alimentary tract by the insect's antioxidants, surfactants, and a high pH in their intestines, which forms a protective peritrophic envelope covering the intestinal epithelium (Barbehenn & Constabel, 2011). The antioxidant capacity of tannins, which by donating electrons prevents the formation of reactive oxygen species, causes a substantial

reduction the genetic alterations and lipid peroxidation within the host cell membranas (Vázquez-Flores et al., 2012) yet causes damaging effects of coccidia (Huang et al., 2018), found that the effect of tannic acid confronted supplied to commercial birds in a field trial with *Eimeria* sp. caused observable improvement of intestinal health (Tonda et al., 2018).

In this study, the hydrolysable tannins had an astringent effect causing tissue contraction damage on coccidia, an effect that has also been reported in other studies (Huang et al., 2018; Sharrajabian & Sun, 2024). It suggests the possibility of using tannin-based agents to counteract infections caused by microorganisms within the enteric tract (Olivas-Aguirre et al., 2015).

This study has shown that tannin extracts obtained from tara pods do have a harmful effect on avian parasites. The inhibitory activity of tannins has been shown to extend to other organisms. For instance, tannins possess an antifungal capacity, particularly on filamentous fungi and post-harvest pathogens (Romani et al., 2021), tannic acid, inhibits bacterial growth and is an alternative or supplement to antibiotics to prevent salmonellosis (Yan et al., 2024). and display antinematodal ovicidal effects against the parasitic worm Haemonchus (Rojo-Rubio et al., 2019). Given these capabilities, tannins appear to be a promising beneficial agent to be used in diverse activities associated to controlling harmful microorganisms in agricultural production.

CONCLUSIONS

This study has shown that the pod extracts of the legume tara possess tannins. Our results have demonstrated that when applied to *Eimeria* coccidia *in vitro* the tannin extracts from tara did compromise the cell membrane integrity and reduce the survival of the parasite. Plant tannins may potentially become an economical and viable way to control *Eimeria* infection in poultry birds. Further studies would be needed to test the effect of tara extracts on *Eimeria* oocysts in vivo and possibly implement its use as bird feed with concomitant recommendations on suitable dosage and frequency of use.

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