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Evaluation of the anticoccidial effect of jataí bee (*Tetragonisca angustula*) propolis extract against *Eimeria* spp. oocysts in broiler

Avaliação do efeito anticoccidiano do extrato de própolis de abelhas jataí (*Tetragonisca angustula*) contra oocistos de *Eimeria* spp. de frangos de corte

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The use of anticoccidial compounds is the primary strategy for controlling avian coccidiosis. The emergence of drug resistance, regulatory restrictions, and societal demands for meat production without the use of antimicrobials have increased the need for natural alternatives to control this disease. Propolis is known for its antimicrobial effects and may be an alternative. In this context, this study aimed to evaluate the anticoccidial activity of jataí bee (*Tetragonisca angustula*) propolis against the sporulation of *Eimeria* spp. oocysts in broiler chicken feces. Propolis ethanolic extracts were tested at 10, 15, and 20% against *Eimeria* oocyst sporulation. Direct use of the extract on oocysts completely inhibited sporulation. When applied to feces, the extract significantly reduced the number of sporulated oocysts. Thus, the jatai bee propolis extract is a promising alternative to commercial anticoccidial agents.
Keywords: *Eimeria* oocysts, jatai bee, propolis.

O uso de compostos antimicrobianos é a estratégia primária para o controle da coccidiose aviária. O aumento da resistência aos compostos comumente utilizados, as restrições regulatórias e as demandas da sociedade pela produção de carne sem o uso de antimicrobianos têm aumentado a necessidade de alternativas de controle naturais para esta doença. A própolis é conhecida por seus efeitos antimicrobianos e pode ser uma alternativa. Neste contexto, este estudo teve como objetivo avaliar o efeito anticoccidiano da própolis de abelhas-sem-ferrão jataí (*Tetragonisca angustula*) contra a esporulação de oocistos de *Eimeria* spp. em fezes de frangos de corte. Extratos etanólicos de própolis foram testados a 10, 15 e 20% contra a esporulação de oocistos de *Eimeria*. O uso do extrato diretamente nos oocistos inibiu totalmente a esporulação. Quando aplicado na presença de fezes de frango de corte, o extrato reduziu significativamente o número de oocistos esporulados. Logo, a própolis de abelha jataí é uma possível alternativa aos agentes anticoccidianos comerciais disponíveis.
Palavras-chave: oocistos de *Eimeria*, abelha jataí, própolis.

1. INTRODUCTION

Coccidiosis is an enteric disease caused by an infection with one or more species of *Eimeria*, an intracellular parasite that multiplies in the epithelial cells of the small intestine, causing severe mucosal damage and impaired nutrient absorption [1]. Coccidiosis has a significant economic impact on the poultry production chain worldwide [2]. This disease occurs after the ingestion of sporulated oocysts of *Eimeria*, which are found in poultry litter, water, and contaminated feed [3]. Sporulated oocysts can survive for long periods outside the host, and non-sporulated oocysts can remain viable for approximately 7 months in the host cecum [2]. Currently, seven species of

Eimeria are known to infect chickens and differ in their pathogenicity, location, and gross lesions: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella* [4].

Eradication of avian coccidiosis is difficult because of increased oocyst resistance and the ability to survive in the environment [5]. The use of anticoccidial compounds is the primary strategy for preventing and controlling this disease. Recently, vaccines and feed additives have been used to improve the management of poultry houses [4, 6]. However, the emergence of drug-resistant *Eimeria* strains, legislative and regulatory restrictions, international trade opportunities, and societal demand for meat production without the use of antimicrobials have forced researchers and poultry companies to develop natural alternatives to control coccidiosis [7].

Propolis is a resinous substance produced by bees through the collection of resins from plants and is altered by the action of enzymes present in their saliva. Despite the differences in its composition, propolis is known for its antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, antiproliferative, and antioxidant properties [8]. Brazil is an important propolis producer and exporter. Propolis obtained from meliponines, also known as stingless bees and native to Brazil, has been studied and is known to contain phenolic compounds; these compounds are responsible for controlling the multiplication of pathogenic microorganisms, improving digestive functions and nutrient absorption, without residues in the meat, and without harming consumer health [9]. The anticoccidial effect of propolis has been evaluated in rabbits and piglets [10, 11], but not in poultry.

In this context, this study aimed to evaluate the anticoccidial activity of jataí bee (*Tetragonisca angustula*) propolis against the sporulation of *Eimeria* spp. oocysts in broiler chicken feces.

2. MATERIALS AND METHODS

2.1 Ethical approval

All experimental procedures were approved by the Ethics Committee on the Use of Animals at the Universidade de Passo Fundo (UPF/Brazil; protocol number 053/2019). All methods were performed following relevant guidelines and regulations.

2.2 Preparation of propolis ethanolic extracts

2.2.1 Raw propolis

Raw propolis of the native jataí bee (*Tetragonisca angustula*) was obtained from an meliponary located in Sarandi (RS, Brazil), in the Alto Uruguai region (27°56'38 S and 52°55'23" W), at an altitude of 503 m. The propolis was packed in sterile plastic bags and sent to the Microbiology laboratory of the Federal Institute of Education, Science, and Technology of Rio Grande do Sul (Sertão, RS, Brazil) and was stored under refrigeration at 4 ºC.

2.2.2 Propolis ethanolic extracts

The methodology used to prepare the extracts was based on techniques previously described by Park et al. (1998) [12] and Zago et al. (2020) [13], with modifications. Samples of 2, 3, and 4 g of propolis were homogenized in a cyclone-type mill. Next, 20 mL of ethanol 40% was added to obtain extracts at concentrations of 10, 15, and 20%. The solutions were submitted to an ultrasonic bath (SB-5200 DTDN; Wincon, Jiangsu, China) with 40 kHz at 21 ºC for 2 h. After extraction, the samples were filtered through quantitative filter paper with 28 µm pores in a vacuum pump. The finished samples were sent to the Laboratory of Biochemistry and Bioprocesses (UPF/Brazil), where they were rotary evaporated at 50 ºC for 30 min to remove the ethanol. The samples were then centrifuged at 956 × *g* for 3 min. Then, the extracts were stored in amber bottles wrapped in aluminum foil and refrigerated at 2 °C until use.

2.2.3 Quantification of total phenolic compounds and flavonoids

The total phenolic compounds and flavonoids were quantified as previously described by Zago et al. (2020) [13]. The total phenolic compound content in the propolis extracts was quantified using the Folin-Ciocalteu method and expressed as gallic acid equivalent (GAE)/g of the extract. The absorbance was measured using a spectrophotometer (Shimadzu, model UV-1800) at 760 nm. The standard curve was prepared with gallic acid ($y = 0.0166x + 0.0049$ and $R^2 = 0.9985$) at concentrations of 10 to 70 µg/mL (Figure 1). Total flavonoids were quantified using the AlCl3 complexation method [14] and expressed as catechin equivalents (CATE)/g of extract. The absorbance was measured at 510 nm using a spectrophotometer. A standard curve was prepared using catechin ($y=0.0046x + 0.0094$, $R^2 = 0.9994$) (Figure 2). All analyses were performed in triplicate.

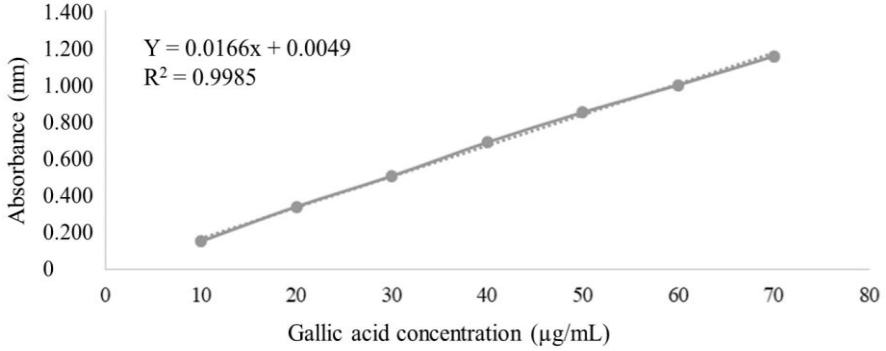

Figure 1: Standard curve of gallic acid used in the determination of phenolic compounds in propolis extract.

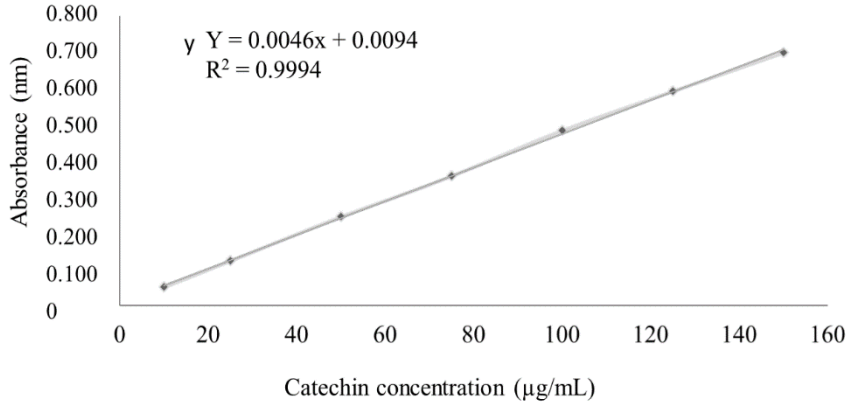

Figure 2: Standard curve of catechin used in the determination of flavonoid compounds in propolis extract.

2.3 Screening for anticoccidial effect of propolis

An initial trial was first carried out with purified *Eimeria* oocysts to determine the anticoccidial effect of propolis extracts of the native jataí bee when used directly in the oocysts.

2.3.1 Oocysts

The *Eimeria tenella* oocysts were obtained and purified at a private laboratory (Ibiúna, São Paulo, Brazil). Oocysts were maintained in sterile water at 4-5 °C to maintain viability but not allow sporulation.

2.3.2 Trial

To prepare samples of propolis ethanol extracts with oocysts, the supernatant was removed and transferred to sterile plastic tubes containing the oocysts according to the following treatments: control (no treatment), 10% propolis extract (T1), 15% propolis extract (T2), and 20% propolis extract (T3). The samples were then incubated in a water bath with minimal aeration, at 29 °C for 24 h to activate the oocyst sporulation process. Subsequently, the sporulated and non-sporulated oocysts were counted under a microscope using a Neubauer chamber. This assay was repeated twice. The concentrations of propolis that exhibited anti-coccidial effects against *E. tenella* oocysts were selected for *in vitro* analyses.

2.4 Screening for anticoccidial effect of propolis

2.4.1 Avian

The experiments were conducted at the Agricultural Extension and Research Centre (CEPAGRO, UPF-Brazil). Ross 308 10-day-old male broilers, were used in this experiment. Animals were housed on a floor covered with new wood shavings with free access to drinking water and fed *ad libitum* with corn- and soybean-based diets without the addition of anticoccidial agents.

2.4.2 Vaccine challenge and quantification of oocysts

A live attenuated vaccine, BioCoccivet (Biovet; Vargem Grande Paulista, Brazil), was used for the vaccine challenge. This vaccine consists of viable suspensions of *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. tenella*, and *E. mitis* isolated from Brazilian flocks.

At 12 days old, the vaccine was inoculated at 0.6 mL per avian using an esophageal cannula attached to a syringe. At 144 h after inoculation, the avians were transferred to cages with trays, and feces were collected. The collected material was refrigerated until further analysis. Sporulated and non-sporulated oocysts were evaluated at the Laboratory of Veterinary Parasitology (UPF, Brazil) through fluctuations in a saturated sucrose solution and visualization under an optical microscope. Quantification of oocysts per gram of feces (OPG) was performed under an optical microscope (40X) according to Gordon and Whitlock [15] and modified by Ueno and Gonçalves [16].

2.4.3 Inoculum preparation and assay

After quantification (123,650 oocysts/g feces) and evaluation of the oocysts (100% non-sporulated), the inoculum was prepared with 50 mL of feces in a 2.5% potassium dichromate solution. The following four treatments were evaluated: control (no treatment), 10% propolis extract (T1), 15% propolis extract (T2), and 20% propolis extract (T3). For each treatment, 2 mL of inoculum and 2 mL of propolis extract were added to a Petri dish at the respective concentrations. Plates were incubated at 28 °C for 24 h. Then, the quantification of sporulated and non-sporulated oocysts was performed as previously described. All assays were repeated thrice.

2.5 Statistics

GraphPad Prism software (GraphPad; San Diego, USA) was used for the analyses, adopting a reference significance level of 5%. Quantification of total phenolic compounds and flavonoids was submitted to analysis of variance (ANOVA) and Tukey's test ($p<0.05$). As the results were not normally distributed (Shapiro-Wilk test), the non-parametric Kruskal-Wallis and Mann-Whitney tests were used to compare quantification means of sporulated oocysts among treatments and sporulated and non-sporulated oocysts within treatments, respectively.

3. RESULTS AND DISCUSSION

More than 200 stingless bee species have been reported in Brazil, approximately 90 of which are endemic. Meliponines belong to the family Apidae and differ from honeybees (*Apis mellifera*) in their social behavior and morphological characteristics of the sting [17-19]. Stingless bee species are widely distributed in Brazil. Among these species, *Tetragonisca angustula*, popularly known in Brazil as the jataí bee, has a great capacity to build nests and survive in different environments, including urban areas [20].

The development of natural feeding additives is required to reduce the use of antimicrobial agents in the animal food production chain. Therefore, propolis extracts have been studied to improve the performance of broiler chickens. The antimicrobial, anti-inflammatory, antioxidant, immunostimulatory, and immunomodulatory effects of propolis extracts have been described [21]. The bactericidal effect of jataí bee propolis extract against several Gram-positive and Gram-negative microorganisms has been previously demonstrated [17, 22].

In addition to its bactericidal effect, propolis has been shown to exert anti-coccidial action against the oocysts of *Eimeria* spp. Infection with *Eimeria* species is widely prevalent in poultry and is considered one of the costliest issues in the production chain. The addition of anticoccidial agents to feed is the main method of controlling coccidiosis. However, the emergence of resistant parasites has led to increased research on the development of new and natural control methods [2, 23]. Studies on propolis extracts, particularly in rabbits, have increased in recent years.

An experiment conducted in New Zealand evaluated the addition of a hydroalcoholic solution of propolis to water and observed a linear reduction in oocysts in rabbit feces [10]. Hollands et al. (1984) [24] tested oral propolis in rabbits and observed a significant reduction in oocysts in the feces and in the intensity of the disease compared to the control group. Hollands et al. (1988) [25] compared propolis with sulfamethazine and sulfaquinoxaline and observed a significant reduction in the intensity of coccidiosis among animals that received propolis compared to those that received coccidiostats. Despite the promising results found in rabbits, few studies have evaluated the action of propolis against the sporulation of *Eimeria* spp. oocysts in broilers. Until now, this is the first study that evaluated the anticoccidial effect of jataí bee propolis extracts.

The results of the quantification of total phenolic and flavonoid compounds are shown in Table 1. The phenolic compound content increased significantly ($p < 0.05$) with augmented propolis extract concentration. Flavonoid concentrations showed significant ($p < 0.05$) differences between propolis extracts at 10%, 15%, and 20%. However, no significant differences ($p > 0.05$) were observed between the 15 and 20% groups.

Table 1: Total content of phenolic and flavonoid compounds in jataí bee (Tetragonisca angustula) propolis extracts.

Quantification (mean ± standard deviation)		
Propolis extract concentration (%)	**Total phenolic compounds concentration (µg/mL gallic acid)**	**Total flavonoid concentration (µg/mL catechin)**
10	20.95 ± 1.07^{a}	5.64 ± 0.70^{a}
15	28.27 ± 0.45^{b}	7.30 ± 0.22^{b}
20	35.18 ± 0.64^{c}	7.81 ± 0.13^{b}

Different lowercase letters in the same column indicate significant differences among the propolis extract concentrations (Tukey's test; $p < 0.05$).

Screening for the anticoccidial effect of propolis demonstrated that the use of propolis extracts at 10 and 15% directly in oocysts completely inhibited sporulation. At 20%, oocyst counting was not possible because the propolis extract ruptured the external and internal layers of the oocyst wall. According to Nogueira-Neto (1997) [26], the propolis stored by jataí bees is extremely viscous compared to other stingless bees, which probably caused the alteration observed in the oocysts. However, considering that 10 and 15% propolis demonstrated anticoccidial effects, these concentrations were selected for the *in vitro* test.

For the screening test, the extract was directly added to the oocysts. Oocysts are easily eliminated by desiccation, sunlight, and heat. However, in the presence of organic matter such as poultry litter and feces, oocysts may become more resistant [27]. Therefore, *in vitro* tests were performed in the presence of chicken feces. The *in vitro* action of propolis on the sporulation of oocysts of *Eimeira* spp. in broiler excreta is shown in Table 2.

Table 2: Action of 10%, 15%, and 20% propolis extract from jataí bees (Tetragonisca angustula) against sporulation of Eimeria spp. isolated from broiler excreta.

Treatment	Mean number ± standard-deviation	
	Number of sporulated oocysts/g of feces	Number of non-sporulated oocysts/g of feces
Control (no treated)	$112{,}783.33 \pm 3{,}969.36^{a,A}$	$0^{a,B}$
Propolis 10%	$88{,}933.33 \pm 2100.20^{ab,A}$	$5{,}250.00 \pm 540.83^{ab,B}$
Propolis 15%	$88{,}116.67 \pm 1654.04^{b,A}$	$5{,}667.67 \pm 76.38^{ab,B}$
Propolis 20%	$88{,}266.67 \pm 809.83^{b,A}$	$6{,}300.00 \pm 100.0^{b,B}$

Different lowercase letters in the same column indicate significant differences among treatments (Kruskal-Wallis test; $p<0.05$). Different uppercase letters in the same line indicate significant differences between sporulated and non-sporulated oocysts (Mann-Whitney U test; $p < 0.05$).

Unlike the screening test, for the *in vitro* test no total inhibition of sporulation was presented, probably because of the presence of organic matter. However, compared to the control group significant differences ($p < 0.05$) were observed in the number of sporulated and non-sporulated oocysts between the control and 15% and 20% propolis extracts.

Quantification showed different amounts of total phenolic and flavonoid compounds, according to the concentration of propolis extract, as follows: 10% (20.95 µg/mL gallic acid; 5.64 µg/mL catechin), 15% (28.27 µg/mL gallic acid; 7.30 µg/mL catechin), and 20% (35.18 µg/mL gallic acid; 7.81 µg/mL catechin). These results indicated that the total phenolic and flavonoid levels increased with elevated extract concentrations. However, no significant differences ($p > 0.05$) were observed among treatments.

The antimicrobial effects of propolis increase poultry resistance to stressors, including heat stress, lead toxicity, and infections with *Eimeria* spp. [28]. In addition to its antimicrobial effects, propolis improves productive performance and physiological parameters. In laying hens, egg mass, and eggshell quality can be enhanced using propolis. By including propolis extract in the diet, the feed intake may increase and improve the body weight of broilers, boosting their growth and function. Other parameters may benefit from propolis intakes, such as decreased mortality rates, carcass characteristics, animal behavior, hematological parameters, enzymes, and the microbiota of the gastrointestinal tract [9, 28, 29]. It has been shown that propolis extract does not represent a risk to animal health, and this compound does not affect intestinal or liver integrity [29].

4. CONCLUSION

A significant reduction in the number of sporulated and non-sporulated oocysts was observed between the control group and the 15% and 20% propolis extracts. Thus, the jatai bee propolis extract is a promising alternative to commercial anticoccidial agents. Further analysis *in situ* is needed to confirm the potential applications of this extract.

5. ACKNOWLEDGMENTS

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