Journal of Applied Biology & Biotechnology Vol. 11(6), pp. 228-232, Nov-Dec, 2023
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2023.135101

Effect of phenolic disinfectant on sporulation inhibition of *Eimeria tenella* for prevention of coccidiosis

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ARTICLE INFO

Article history:
Received on: March 30, 2023
Accepted on: July 11, 2023
Available online: October 25, 2023

Key words:
Coccidiosis,
Disinfectants, Poultry,
Eimeria,
Sporulation.

ABSTRACT

Caecal coccidiosis in broiler chickens is caused by *Eimeria tenella*, a parasite that causes severe mortality and huge economic loss. *E. tenella* exists in two stages, namely, sporulated and unsporulated stages, and the infective sporulated stage causes infection and leads to caecal coccidiosis. An *in vitro* study was carried out to understand the effect of phenolic disinfectant (PD) on the sporulation suppression of *E. tenella* oocyst. Unsporulated oocysts of *E. tenella* from infected broiler birds were used for the experiments. The groups consisted of control (C, saline), negative control (NC, sodium hydroxide), positive control (PC, ammonium hydroxide), T1 (PD at 5 mL/L), and T2 (PD at 10 mL/L). The percentage of oocysts that were unsporulated, sporulating, and sporulated was quantified, and then, the inhibitory activity (IA) was calculated. A higher percentage of sporulated oocysts was observed in C (71%) and NC (65%). Groups such as PC, T1, and T2 showed 3.89%, 38.44%, and 9.33% of sporulated oocysts, which were found to be lesser than C and NC. The IA of the groups was observed to be 2.96%, 91.30%, 52.25%, and 70.58% for NC, PC, T1, and T2, respectively ($P < 0.05$). Based on these results, we infer that PD suppresses *E. tenella* sporulation and could be used to prevent coccidiosis in poultry farms.

1. INTRODUCTION

Coccidiosis is a parasitic disease caused by *Eimeria* species in chickens costing a worldwide annual loss of more than a billion US dollars [1]. Chickens are affected by seven different *Eimeria* species and amongst them; *E. tenella* is the most pathogenic [2-4]. The life cycle of *Eimeria* involves many stages occurring inside and outside the host [5]. This parasite exists in two stages: Sporulated and unsporulated stage. The sporulated stage causes infection in the birds. Dormant *Eimeria* oocysts undergo a sporulation process to form sporulated oocysts, which are considered pathogenic [6]. The sporulation of the oocysts happens outside the birds and is dependent on temperature, humidity, and access to oxygen [7].

The infection is transmitted to the birds through the ingestion of infective sporulated oocysts that target the gut [1]. After targeting the gut, the intestinal epithelial cells are invaded during the multiplication stage, destroying the intestinal lining [5]. This negatively impacts performance, including increased feed conversion values and reduced body weight gain, leading to mortality [1]. The oocyst cell wall of coccidia is reported to be robust and highly resistant to chemical treatments [8]. Since the infectious nature of the oocyst is decided outside the host, it is possible that by inhibiting the sporulation process, the infectivity of the coccidia could be lessened.

Disinfectants, classified under the biosecurity program, are used to prevent the incidence of disease caused by pathogenic organisms [9]. These products are used to clean and disinfect the farm floor, equipment, cages, and associated accessories. Several studies have reported the efficacy of different classes of disinfectants, reagents, and herbal extracts against *Eimeria* oocysts [9-11]. In a study reported by Gadelhaq *et al.,* chemicals such as ethanol, formalin, and sodium hypochlorite inhibited the sporulation ranging from 49% to 100% [9]. William *et al.* showed the sporulation inhibitory effect of phenols, chlorinated phenols, ammonia, and methyl bromide [11]. In another study, chemicals such as acetic acid, cresols, and a combination of benzene and xylene were found to be good inhibitors of *E. tenella* sporulation [12]. The efficacy of phenolic disinfectant (PD) comprising a mixture of cresylic acid, ortho-benzyl-para-chlorophenol, and ortho-phenyl phenol against sporulation suppression of *E. tenella* is not known. The objective of the study was to test the efficacy of PD on the inhibition of *E. tenella*'s sporulation.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Ammonium hydroxide, sodium hydroxide, sodium chloride, and potassium permanganate were purchased from Sigma-Aldrich, India. The PD comprising a mixture of a cresylic acid (30%), ortho-benzyl-para-chlorophenol (3%), and ortho-phenyl phenol (4%) was received from Kemin Industries South Asia Pvt. Ltd., Chennai, India.

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2.2. *E. tenella* Oocysts Collection and Purification

Oocysts of *E. tenella* were taken from commercial broiler birds affected with clinical coccidiosis. The symptoms of clinical coccidiosis are bloody diarrhea, reduced feed intake, and reduced growth rates, and in severe cases, it leads to mortality [13,14]. Clinical coccidiosis in broiler birds is diagnosed by monitoring the presence of gross lesions in the intestinal tract which includes thickening, hemorrhage, and ulceration [15,16]. Caecal contents were cleaned by flotation method using a saturated sodium chloride salt solution [9]. Briefly, 5 g of caecal contents from the infected birds were collected and mixed with 50 mL of saline [17]. The mixture was vortexed for 1 min and the resulting suspension was filtered using a double-layered muslin cloth and centrifuged at 1000 g for 5 min [17]. After removing the supernatant, the pellet was re-suspended in 5 mL of saturated sodium chloride salt solution [18]. Oocysts float in saturated salt solutions forming an opaque ring on the top, thereby enabling us to collect easily [17,18]. Then, the collected oocysts were pooled and washed 3 times with phosphate-buffered saline using a centrifugation process (1000 g, 15 min, 25°C, Eppendorf, 5810R). The oocysts were counted using a hemocytometer (BS748, Rohem, India) and the morphological characteristics were considered for the confirmation of the oocysts stage [19].

2.3. Treatment Groups

The study consisted of three control groups and two treatment groups. The control groups are control (C), a negative control (NC), and a positive control (PC). The C, NC, and PC groups were treated with saline, sodium hydroxide, and ammonium hydroxide, respectively. The C group is treated with saline, which helps in assessing the viability of the oocysts, during the treatment period. In NC, sodium hydroxide is used, which was known to have no effect on the sporulation of oocysts [19]. In PC, ammonium hydroxide is used and is known to inhibit the sporulation process of oocysts [19]. The treatment groups were designated as T1 and T2, treated with PD at 5 mL/L and 10 mL/L, respectively. After the treatment, the oocysts were subjected to sporulation for 7 days.

2.4. Oocyst treatment

The effect of PD on *E. tenella* oocysts was studied using unsporulated oocysts, as described by Gadelhaq *et al.,* with modifications [9]. Briefly, the oocysts were normalized to the concentration of 10^5 cells/mL using saline. To 1 mL of cells, chemicals or PD were added at the concentration mentioned in Table 1 in triplicates and incubated at room temperature for 3 h. The cells were then centrifuged at 8000 g for 15 min at 25°C, and the supernatant was discarded. Then, the oocyst pellet was washed 3 times with 1 mL of saline. After washing, the samples were subjected to sporulation using 1 mL of 2.5% $KMnO_4$ and aerated for 7 days using SeBO® air pump AP-500 (AquaSovi, India). Samples were collected (25 µL) from each group on daily basis and immediately analyzed for the sporulation stage using a hemocytometer and Leica DM-1000 microscope (Leica Microsystems, Germany) at 40X magnifying power. At the end of 7 days, the percentage of cells which were sporulated, sporulating, and unsporulated was calculated based on the initial cell count.

Table 1: Description of the treatment groups and their dosage.

Treatment groups	Products/reagents	Dose*
Control	Saline	-
Negative control	Sodium hydroxide (10 N)	0.1 mL/mL
Positive control	Ammonium hydroxide (33%)	0.5 mL/mL
T1	PD	5 mL/L
T2	PD	10 mL/L

*Doses were fixed based on the recommended dose of the product and concentrations used in the reported studies. PD: Phenolic disinfectant

2.5. Inhibitory Activity (IA) Measurement

Based on the sporulation stage of cells calculated above, the IA of the product and chemicals was based on the calculation shown in Equation 1, described by Gadelhaq *et al.,* and Samaha *et al* [9,20].

Calculation of IA:

$$IA\ \%=\frac{\%\ \text{Sporulation of control}\ -\%\ \text{Sporulation of treatment}}{\%\ \text{Sporulation of control}}\times 100$$

(Equation 1)

2.6. Statistical Analysis

The Statistical Analysis System from STATGRAPHICS® Centurion XVI software, Version 16.2.04 (Statgraphics Technologies, Inc., Virginia, USA) was used for performing the statistical analyses. Data were analyzed with one-way ANOVA at a 95% confidence level and $P < 0.05$ was considered for determining the significant difference.

3. RESULTS AND DISCUSSION

3.1. Identification of Different Stages of the Oocyst

After treatment with the respective products, the cells were identified based on their morphology, and the results are shown in Figure 1 [19]. A clear differentiation between sporulated, sporulating, and unsporulated stages was observed. Unsporulated oocysts appeared as one whole cell inside the oocyst wall (Figure 1a). The sporulating oocysts were identified with three to four divided cells, overlapping each other inside the oocyst (Figure 1b). The sporulated stage showed well-defined, three to four distinct cells inside the oocyst (Figure 1c). In all the groups, we could identify the various stages of oocysts, which had a clear differentiation of cells within the oocyst (Figure 1).

Kasim *et al*., also performed similar studies for assessing the effect of *Rosmarinus officinalis* on the sporulation inhibition of *E. tenella* [21]. In this report, different stages of oocysts were reported with clear differentiation of cells within the oocysts, as observed in this study.

Li *et al.,* investigated the effects of nitric oxide (NO) donors on the sporogony of *E. tenella* oocysts and the blocking effects of NO scavenger, hemoglobin (Hb), against NO donors [22]. The study found that acidic sodium nitrite, S-nitrosoglutathione, and S-nitroso-N-acetylpenicillamine were able to completely inhibit the sporulation of the oocysts in a dose-dependent and time-dependent manner. However, in this study, sodium nitroprusside did not demonstrate the desired effects, suggesting the compounds plays a major role in the sporulation suppression.

In general, all freshly shed oocysts consist of a thickened outer well and a rounded mass with a nucleated zygote, and this structure remains intact until the sporulation process occurs [23]. In our study, such morphology of the oocyst was considered as the unsporulated oocyst. In *Eimeria* species, once the sporulation process occurs, the distinguishing characteristic of each species becomes more apparent. For instance, in the final stages of sporulation, four sporocysts develop within the circumplasm of the oocyst, each containing two

banana-shaped sporozoites, and such cells are identified as sporulated oocysts [24]. The sporozoites within the sporulated oocyst may vary in shape and size depending on the species. Before the formation of the sporocysts, the unsporulated oocyst contains a diploid sporont stage which further develops by meiosis, and these oocysts cells are in the sporulating stage, which then divides into four sporocysts [25].

Cysteine proteases (CPs) are enzymes that cleave peptide bonds using a cysteine residue in their active site. They are expressed by many protozoan parasites including *E. tenella* and are considered to be major virulence factors due to their roles in parasite invasion, replication, and immune evasion [26]. The CP-dependent activities were present throughout the sporulation process suggesting that CPs are active at various stages of the parasite life cycle [27]. This is consistent with the reported studies that CPs as important virulence factors that are involved in many aspects of the parasite life cycle. As various stages of sporulation were seen in this study, it could be possible that the CP was inhibited by the tested compounds leading to sporulation suppression.

3.2. Effect on Sporulation and IA

After treatment of oocysts, the percentage of sporulated, unsporulated, and sporulating oocysts were calculated and the results are shown in Figure 2. It was observed that each treatment group showed varying percentages of oocyst in each of the three stages. More than 65% of oocysts were in a sporulated stage in C and NC, whereas treatment groups showed less percentage of sporulated oocysts. Among the groups, sporulating oocysts were found to be higher with saline and NC. Jitviriyanon *et al.* also found that the sporulating percentage of oocysts in the saline-treated group was more than 65%, which corroborates the findings observed in this study [28]. The highest percentage (91%) of unsporulated oocyst was observed in the PC group. Chroustova and Pinka also conducted similar kinds of experiments with ammonium hydroxide as one of the test groups and the unsporulated oocysts were found to be >98% [29]. There was a difference in efficacy between this study and reported by Chroustova and Pinka, which could be due to the difference in the number of cells taken for the study [29]. However, >90% of unsporulated oocysts were observed in this study and reported by Chroustova and Pinka [29]. A similar study was also performed with ammonium hydroxide (125 µg/mL) and the unsporulated oocysts were reported to be <5% and the sporulated oocysts were 65% [28]. Such difference in efficacy was inferred to be concentration linked. The concentration of ammonium hydroxide tested by Samaha *et al*., and by Chroustova and Pinka was at a high concentration (5%), whereas Jitviriyanon *et al.* tested ammonium hydroxide at 0.0125% [20,28,29]. Such an effect could be resulting from the binding ability between hydroxyl ions and lipids in the cell membrane, the saponification of the lipid leads to the dysfunction of the cell membrane [30]. In NC, the unsporulated oocysts were found to be around 6% indicating sodium hydroxide that did not exhibit any effect on the sporulation process. The results of the sporulation experiments with PC and NC were found to be very closer to the results reported earlier [29]. This validates the sporulation experiments that were carried out for this study.

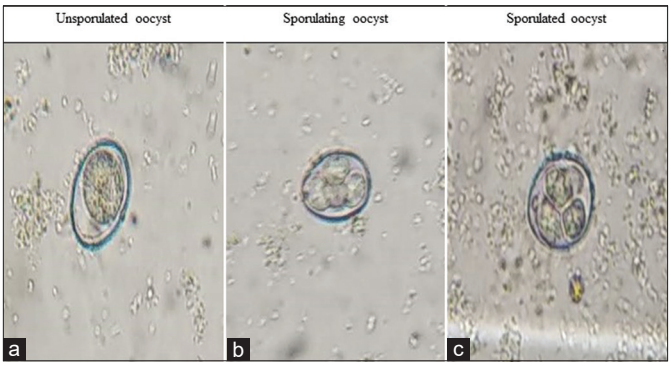

Figure 1: (a-c) Differentiation of sporulation of *Eimeria* oocysts visualized under a microscope at ×40. Images were captured during sporulation analysis.

The test groups, T1 and T2, also showed a high percentage of 55% and 71% unsporulated oocysts, respectively. In a study reported by the author, You 30% cresol was taken for the study and the sporulation rate was found to be between 20% and 85% [12]. Higher concentrations showed less sporulation rate indicating the inhibition of the sporulation process and lower concentrations showed a higher sporulation rate suggesting less effect on inhibiting the sporulation process. Since, PD contains a mixture of saponified cresol, ortho–benzyl-para-chlorophenol, and ortho-phenyl phenol, the results observed in this study agree with the results reported by the author, You [12]. Furthermore, a dose-dependent increase in the unsporulated stage was observed between T1 and T2, signifying the impact of PD on the interference of sporulation. In addition, to the high percentage of sporulated oocysts in C and NC, the number of sporulating oocysts was also high and tends to become sporulated oocysts.

In a study conducted by Del Cacho *et al.,* with artemisinin on the sporulation of *E. tenella*, reduced sporulation rates were observed [1]. Such reduced sporulation rates were inferred to affect the reproduction of this parasite by altering the *E. tenella* oocyst wall [1]. Fatemi *et al.*

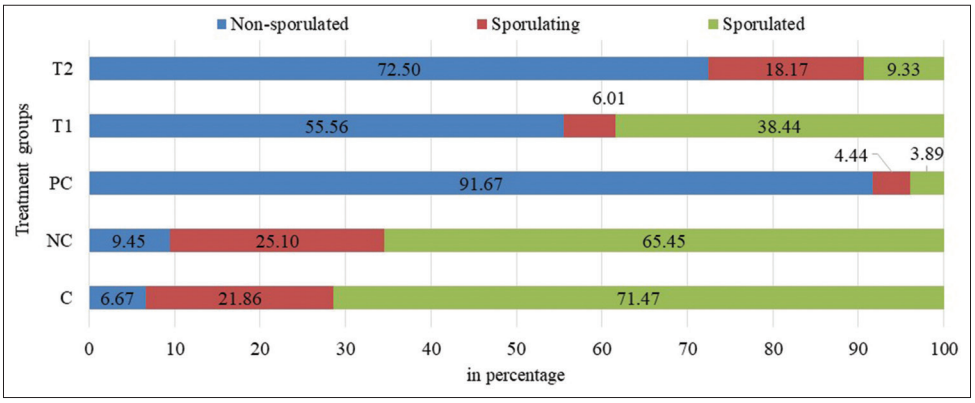

Figure 2: The percentage of oocysts in the respective sporulation stage at the end of the treatment period. Each data represents the mean of triplicates. C: Saline; NC: Sodium hydroxide; PC: Ammonium hydroxide; PD: Phenolic disinfectant; T1: PD-5 mL/L; T2: PD-10 mL/L.

also showed that sporulation of mixed oocysts of *Eimeria* species was inhibited on treatment with *Artemisia annua* [31].

The sporulation inhibition effect of *Phyllanthus emblica* against *E. tenella* was tested by Sharma *et al* [32]. In this study, the oocysts were treated with various concentrations of *P. emblica,* and an almost 35% reduction of sporulated oocysts was observed. Boyko *et al.*, reported the activity of various plant species having essential oils toward sporulation inhibition of *Eimeria* [33]. The results were essential oil from *Syzygium aromaticum* exerted a 54% killing effect on the sporulated oocysts and the sporulated oocysts presence was found be zero [33]. These findings suggest that the essential oil exerted its action through the alteration of the cell wall and sporulation process.

The groups were evaluated for IA and the results are shown in Table 2. IA results give the overall view on the efficiency of the reagent or product that was tested in this study, as it considers the sporulating cells tending to become a sporulated stage. It was observed that the highest IA of 91.30% was observed for PC followed by T2 (70.58%), T1 (52.25%), and NC (2.96%), and was found to be statistically different between each of the groups ($P < 0.05$).

Table 2: Inhibitory activity of the treatment groups.

Treatment groups	IA (%), (mean±SD, n=3)
NC	2.96±1.44^{d}
PC	91.30±3.04^{a}
T1	52.25±2.47^{c}
T2	70.58±2.42^{b}

Statistical difference between the treatment groups is represented by different alphabets in superscripts ($P<0.05$). NC: Sodium hydroxide; PC: Ammonium hydroxide; PD: Phenolic disinfectant; T1: PD-5 mL/L; T2: PD-10 mL/L

In an *in vitro* study conducted by Abouelenien *et al.*, with 10% crude watery extract of *Psidium guajava*, the IA was found to be 86.4% [34]. *Psidium guajava* was also tested in the litter, in the manure of birds under field conditions, and reported to decrease the sporulation percent of coccidial oocyst [34]. Such sporulation inhibition resulted in reduced infection rates in the broiler birds.

In another study conducted by Daugschies *et al.*, the efficacy of the cresol-based disinfectant varied between 17% and 49% for various types of coccidia [35]. Cresol derivatives such as chlorocresol were evaluated for their IA by El sherry *et al* [36]. At 10% and 20%, the IA was found to be 86.7% and 92.7%, respectively. In this study, the PD at 10mL/L (1%) showed IA of 70.58%. Daugschies *et al.* (2007) showed oocyst destruction after a contact time of 90 min or more with the cresol-based products [35]. Williams indicated that the efficacy of the disinfectant was superior toward unsporulated oocysts compared to sporulated ones [11].

These preliminary results suggest that PD has the potential to prevent coccidiosis incidence in broiler farms.

4. CONCLUSION

Although drugs are available to treat coccidiosis, it has disadvantages such as acquiring resistance, residue concerns in meat, and incompatibility with other chemicals. In broiler farms, the disinfection process is followed regularly, and by choosing the appropriate disinfectant formulation, the coccidiosis incidence in broiler chickens could be prevented. The results that were reported in the study suggest that PD could inhibit sporulation of the oocysts and thereby, coccidiosis can be circumvented to a larger extent. By adopting this practice, the usage of antibiotics in broiler feed can be reduced.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

6. FUNDING

There is no funding to report.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data generated in this study are included in this article.

10. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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How to cite this article:
Rajendran RM, Fatima SN. Effect of phenolic disinfectant on sporulation inhibition of *Eimeria tenella* for prevention of coccidiosis. J App Biol Biotech. 2023;11(6):228-232. DOI: 10.7324/JABB.2023.135101