AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

in vitro Effect of organic acid combinations on sporulation of *Eimeria tenella*

Suttitas Tongkamsai[a,*], Siraprapa boobphahom[b], Panjaporn Phothong[c], Piyawan Boonkerd[d], Somchai Sompaisarnsilp[a]

[a] *Faculty of Veterinary Science, Rajamangala University of Technology Tawan-Ok, Chonburi 20110, Thailand*

[b] *Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand*

[c] *Alternative to Antibiotics (ATA) Research Unit, Rajamangala University of Technology Tawan-Ok, Chonburi 20110, Thailand*

[d] *Smart Vet Co., Ltd., Thailand*

Article Info

Article history:
Received 22 August 2024
Revised 13 January 2024
Accepted 23 January 2025
Available online 28 February 2025

Keywords:
Chicken,
Eimeria tenella,
Inhibition,
Oocyst,
Sporulation

Abstract

Importance of the work: Organic acids show potential for inhibiting *Eimeria tenella* that causes chicken coccidiosis with diarrhea and weight loss; however, there is no available specific efficacy data.

Objectives: To evaluate citric acid and formic acid, both individually and combined, as well as a commercial product (known as Bluetec) for *in vitro* inhibition of *E. tenella*.

Materials and Methods: *E. tenella* oocysts were identified using morphology and polymerase chain reaction. The half maximal inhibitory concentration (IC50) for sporulation inhibition was determined by exposing oocysts to citric and formic acids at various concentrations. Trials consisted of citric acid, formic acid, their combination and Bluetec, with sporulation inhibition assessed after 24 hr and 48 hr by counting unsporulated and sporulated oocysts.

Results: The IC50 values were 4.33 mg/mL for citric acid and 2.40 mg/mL for formic acid. After 24 hr of incubation, the sporulation inhibition (SI) percentages were 2.95% for the 3% diclazuril (PC), 12.07% for citric acid, 8.50% for formic acid and 15.13% for the combination and 48 hr of incubation were 21.49%, 11.34% and 23.51%, respectively. Incubation for 48 hour resulted in a reduced sporulation percentage, indicating that the inhibition of sporulation was dependent on the duration of exposure. The SI percentages were 0% for distilled water, 7.96% for PC and 29.74% for Bluetec, highlighting significant differences among the tested items and suggesting a synergistic effect between citric and formic acids.

Main finding: Combining citric acid and formic acid, as well as using Bluetec effectively inhibited *E. tenella* sporulation. There was a notable synergistic effect of citric and formic acids, though its mechanism remained unclear. The most effective treatments were citric and formic acids with incubation for 48 hr and Bluetec with incubation for 24 hr. These organic acids could be utilized as food additives or disinfectants in poultry production.

* Corresponding author.
E-mail address: Suttitas_to@rmutto.ac.th (S. Tongkamsai)

https://doi.org/10.34044/j.anres.2025.59.1.08

Introduction

Chicken coccidiosis, caused by protozoa of the genus *Eimeria*, damages the intestinal lining, leading to digestive problems, reduced food conversion efficiency and high mortality rates, resulting in significant economic losses for poultry farms (Mesa-Pineda et al., 2021). *Eimeria* species are found globally, including in Thailand (Kaewthamasorn et al., 2015). In chickens, seven distinct species have been documented: *E. tenella, E. acervulina, E. maxima, E. necatrix, E. mitis, E. brunetti* and *E. praecox*. Notably, *E. tenella* is among the most virulent species, explicitly targeting the ceca of chickens and leading to hemorrhagic conditions (Mathis et al., 2024). Traditionally, the identification of *Eimeria* has relied on evaluating oocyst morphology using microscopy. However, implementing molecular techniques has enhanced accuracy, minimizing the risk of misdiagnosis among species with similar morphological features (Gyorke et al., 2013). Under warm, humid litter conditions, thousands of unsporulated oocysts sporulate within 48 hr, becoming infectious (Soulsby, 1986). Infection by sporulated *E. tenella* in chickens has led to intestinal villi damage, resulting in reduced nutrient absorption and overall performance (Williams, 1998). Their rapid development and resilient, thick-walled oocysts facilitate widespread coccidiosis transmission through poultry litter and soil particles (Mai et al., 2009). While oocysticides, such as ammonia, methyl bromide and carbon disulfide, as well as phenolic products are effective, their toxicity limits practical use. Therefore, targeting sporulation is crucial for control (Stephen et al., 1997).

Organic acids are naturally occurring compounds considered relatively safe for inclusion in poultry feed, especially compared to synthetic chemical agents. These acids have been reported to exhibit antimicrobial properties, enhance immune responses and serve as growth promoters in broiler chickens (Khan et al., 2022). Numerous researchers have tested the anticoccidial effects of different substances against *E. tenella* using assays inhibiting oocyst sporulation. For example, organic acids, such as mixtures containing malic acid, have been demonstrated to decrease the efficacy of *E. tenella* and *E. bovis* infections by lowering pathogen virulence and lessening inflammatory responses in the host (Balta et al., 2021), with chemicals such as acetic acid and benzene-xylene combinations proving effective (You, 2014). However, the efficacy of citric acid, formic acid and their mixtures in inhibiting *E. tenella* sporulation has not been thoroughly evaluated.

The results from the study *in vitro* on the inhibition of *Eimeria* oocyst sporulation using organic acids enable researchers to control and manipulate specific variables, ensuring an accurate assessment of the direct effects of these compounds without interference from other factors present in a chicken. Therefore, the current study investigated the *in vitro* anticoccidial effects on chicken coccidia of citric acid and formic acid and their combinations.

Materials and Methods

Parasites

Cecal samples from naturally infected broiler chickens were collected from farms in Sa Kaeo province, Thailand and transported to the Alternative to Antibiotics (ATA) Research Unit at Rajamangala University of Technology Tawan-Ok. Species identification was based on morphological characteristics outlined by Thienpont et al. (1979) and confirmed using polymerase chain reaction. Often, *Eimeria* species can be distinguished by examining the shape and size of the oocysts under a microscope. Typically, *E. tenella* oocysts are ovoid, measuring approximately 19 µm - 16 µm (Mares et al., 2023). DNA extracted from the samples was used to amplify the ITS-1 region (Fig. 1), as described by Haug et al. (2007). The oocysts were concentrated, collected in 2.5% potassium dichromate and stored in a refrigerator at 2–5°C until further use.

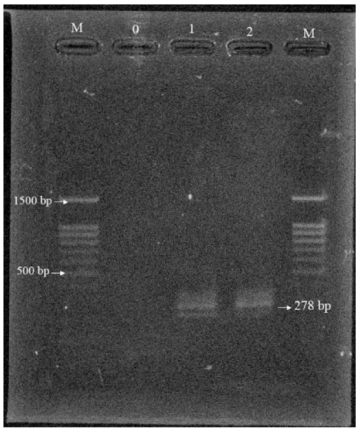

Fig. 1 Polymerase chain reaction (PCR) amplification of *Eimeria tenella* DNA showing a 278 bp product. Lanes M: DNA ladder (100 bp); Lane 0: negative control; Lane 1: positive control; Lane 2: sample. The expected band at 278 bp confirms the presence of *E. tenella*.

Chemicals and organic acid mixtures

The organic acids were citric acid anhydrous (QReC; New Zealand) and formic acid (Fischer Chemical; UK). The commercial product used is known throughout this manuscript as Bluetec and contains 0.2 mg/ml citric acid and formic acid 0.12 mg/ml. It was received from Smart Vet Co., Ltd., Thailand.

Determination of the inhibition concentration 50%

The half maximal inhibitory concentration (IC50) of each organic acid was assessed individually and then their effects were evaluated when combined in mixtures. The experiment used 24-well cell culture plates, with each organic acid tested in triplicate. Oocyst counting was performed using a Neubauer hemocytometer as the reference method (Conway and McKenzie, 2007). Each well was added with 1 mL of a pre-incubated oocyst suspension (n = 15,000) and 1 mL of organic acid and incubated for 24 hr. Subsequently, 10% organic acid (the initial concentration) was tested using five two-fold serial dilutions, resulting in final concentrations of 1% (10 mg/mL), 0.5% (5 mg/mL), 0.25% (2.5 mg/mL), 0.125% (1.25 mg/mL) and 0.0625% (0.625 mg/mL). The IC50 was calculated from curves plotted of the number of oocysts against the concentration of the component, identifying the concentration at which the oocyst count was reduced to one-half of its initial value (Gessner, 1995).

Effect of organic acid mixtures on E. tenella oocyst sporulation

The oocysts were harvested using a flotation technique with a saturated sodium chloride (NaCl) solution. To prepare for sporulation, the salt solution was removed by washing the oocysts with distilled water and centrifuging 3–4 times at 1,500 revolutions per minute for 5 min in 50 mL centrifuge tubes. Afterward, 15 mL of distilled water were added to the salt-free oocyst suspension and the oocyst concentration per milliliter was calculated. For the experiment, 100 oocysts were added to dishes containing citric acid or formic acid and left at room temperature with exposure to oxygen (Table 1). The oocysts were observed using a hemocytometer at 24 hr and 48 hr to determine the sporulation rate, with the percentages of sporulated oocysts recorded. Controls were also included in the study. The negative control used distilled water, which does not inhibit oocyst sporulation, while the positive control used PC, which effectively prevents sporulation. Bluetec was incubated for 24 hr, which was consistent with the incubation period used for both controls. After treatment, all oocysts were allowed to sporulate by exposing them to pumped air for 72 hr (Williams, 1997). Finally, the sporulated oocysts were examined under a light microscope at 20× magnification (Fig. 2). Sporulated oocysts were counted and the percentage of sporulation inhibition (SI) was calculated using Equation 1 and 2:

$$\text{Sporulation (\%)} = \frac{\text{Number of sporulated oocysts}}{\text{Total number of oocysts}} \times 100 \quad (1)$$

$$\text{SI (\%)} = \frac{\text{Sporulation \% of Control - Sporulation \% of Treated}}{\text{Sporulation \% of Control}} \times 100 \quad (2)$$

where SI (%) is the percentage of inhibition of the sporulation.

Table 1 Description of treatment groups

Treatment group	Mixture/product	Recommended dose	Used dose
NC	Distilled water	-	-
PC	3% Diclazuril	2–3 mg/mL	2.5 mg/mL
Ca	Citric acid	-	0.4 mg/mL
Fa	Formic acid	-	0.2 mg/mL
Ca + Fa	Citric acid + Formic acid	-	0.4 mg/ml, 0.2 mg/mL
BT	Bluetec	1 ml/L	1 ml/L

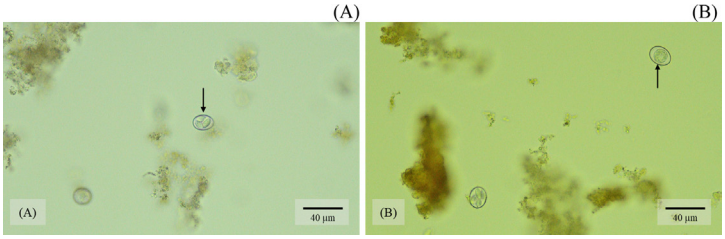

Fig. 2 Characteristics of *Eimeria tenella* oocysts: (A) sporulated oocyst (black arrow); (B) unsporulated oocyst (black arrow).

Statistical analysis

Statistical analyses were conducted using the GraphPad Prism (version 9.0; GraphPad Software Inc., La Jolla, CA). The data were presented as mean ± SD values. Statistical significance was determined at $p < 0.05$ using analysis of variance (ANOVA), followed by Tukey's multiple comparisons test to identify differences between means.

Results

The IC50 value for each organic acid is shown in the logarithmic dose-response curve (Fig. 3). The IC50 was not determined for the Bluetec product, as it did not achieve at least 50% inhibition of *E. tenella* oocyst sporulation. After 24 hr of incubation, the IC50 values for citric acid and formic acid were 4.33 mg/mL and 2.40 mg/mL, respectively.

Following the treatment of oocysts, the percentages of unsporulated oocysts and inhibition of sporulation were calculated, with the results shown in Table 2. Over 75% of oocysts were sporulated in the negative and positive controls, while the treatment groups had less than 70% sporulated oocysts. After 24 hr of incubation with PC, the unsporulated oocysts of *E. tenella* had a percentage of unsporulated oocysts of approximately 20%. In contrast, oocysts incubated with citric acid and formic acid and the combination of citric and formic acids had varying levels of sporulation. Specifically, after 24 hr of incubation with the two treatments, high percentages of unsporulated oocysts were observed (29.64% for citric acid and 25.73% for formic acid). As incubation continued to 48 hr, the percentages of unsporulated oocysts for each treatment tended to increase, reaching 36.60% for citric acid and 28.56% for formic acid, suggesting the enhanced inhibitory effects associated with prolonged exposure. Furthermore, the extension of the incubation time using citric acid and formic acid from 24 hr to 48 hr seemed to enhance sporulation inhibition, with increases from 12.07% and 8.50% to 21.49% and 11.34%, respectively. After 24 hr and 48 hr of incubation with the combination of citric and formic acids, the percentages of unsporulated oocysts of *E. tenella* (32.22% and 39.87%, respectively) increased significantly compared to those treated with citric acid or formic acid alone, indicating that the combination enhanced efficacy against *E. tenella* and likely synergistically inhibited sporulation.

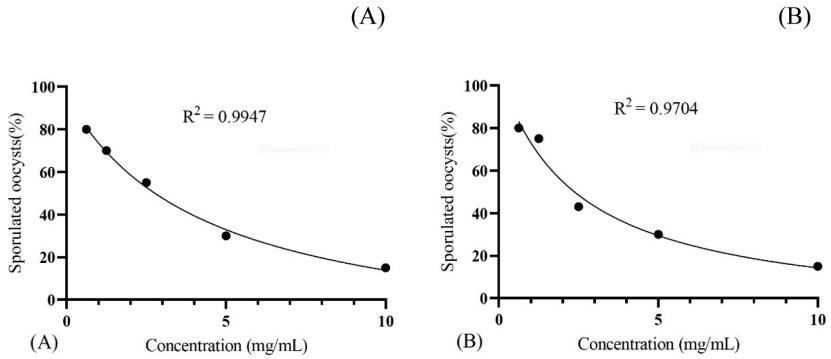

Fig. 3 Logarithmic dose-response curve of percentage inhibition of *Eimeria tenella* sporulation by organic acids: (A) citric acid; (B) formic acid. R^2 = coefficient of determination.

Table 2 Effect of citric acid, formic acid for different incubation times on sporulation (mean ± SD) percentage of *Eimeria tenella* oocysts

Item	NC	Citric acid		Formic acid		Citric + Formic acids		PC
	24 hr	24 hr	48 hr	24 hr	48 hr.	24 hr	48 hr	24 rh
Unsporulated oocyst (%)	9.46±1.08^{a}	29.64±1.12^{c}	36.60±0.33^{A}	25.73±0.43^{d}	28.56±0.69^{B}	32.22±0.44^{e}	39.87±1.59^{C}	20.70±0.11^{b}
Sporulation inhibition* (%)	0	12.07±1.37	21.49±0.89	8.50±1.65	11.34± 0.89	15.13± 1.16	23.51± 0.59	2.95±2.02

NC = negative control; PC = positive control.

Mean±SD in the same row with different superscripts differ significantly ($p < 0.05$); lowercase letters indicate differences among mean values measured at 24 hr, while capital letters indicate differences among means measured at 48 hr; *No significant differences were detected in the percentage of sporulation inhibition.

The tests were assessed for their percentage of inhibition of sporulation, with the results shown in Fig. 4. Oocysts incubated for 24 hr in PC (positive control) and Bluetec exhibited incomplete sporulation. Both treatments significantly reduced the sporulation ability of *E. tenella* compared to distilled water (negative control). Notably, Bluetec produced the highest SI (29.74%), which was significantly greater than the SI value for the positive control (PC) (7.96%).

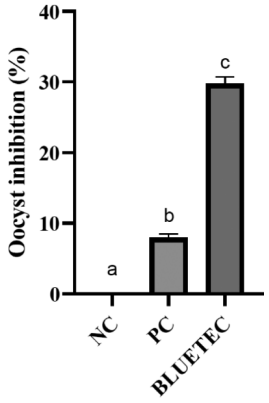

Fig. 4 Effect of Bluetec on inhibition of *Eimeria tenella* oocysts *in vitro* compared to negative control (NC) and positive control (PC), where different lowercase letters above bars indicate significant differences ($p < 0.05$), and error bars indicate ± SD.

Discussion

Under field conditions, the frequent incidence of mixed infections complicates achieving a precise diagnosis (Long and Joyner, 1984). Despite this, *E. tenella* remains notably invasive compared to other species and has been reported to be the most prevalent (Ojimelukwe et al, 2018). The study utilized molecular techniques, which successfully identified *E. tenella*. The sporulation of *Eimeria* oocysts in litter and their ingestion by poultry are critical factors in the epidemiology of coccidiosis, as an oocyst that has not undergone sporulation cannot infect the host, even if ingested in large quantities (Felici et al., 2021). In the current study, sporulation inhibition was used as a critical criterion to assess the effectiveness of the tested organic acids. The distinct characteristics of *Eimeria* become apparent after sporulation, at which point it becomes capable of causing infections (Conway and McKenzie, 2007). You (2014) evaluated the anticoccidial effects of organic acids compared to chemical alternatives, indicating that these acids are promising candidates for suppressing *E. tenella* oocyst sporulation. The current study showed that mixtures of organic acids reduced the *in vitro* sporulation of *E. tenella*., with the results agreeing with another report that combining organic acids was more effective than single compounds in controlling bacterial infections (Toschi et al., 2020). The effectiveness of citric and formic acids was evaluated using two parameters: 1) incubation time and 2) combinations of the two acids. Based on the current results, there was inhibition of sporulation, with the percentage of unsporulated oocysts being approximately 25−29% after 24 hr, increasing to 28−36% after 48 hr. Limited knowledge exists concerning the mode of action of acids against parasites. However, Abbas et al. (2011) reported that acids act against microbial cells by releasing protons (H+) within the cell, which inhibits the function of vital enzymes and forces the cell to expend energy to expel the excess protons, ultimately leading to cell death. Other researchers have stated that organic acids may exhibit an anti-sporulation effect either by causing a toxic accumulation of anions in the cell's cytoplasm (Mani-López et al., 2012) or by interfering with the oxygen availability required for sporulation (Zaman et al., 2012). However, the capacity of organic acids to penetrate the resistant oocyst wall and the variation in their molecular size are not well understood and need further investigation. The ability of the combination of citric and formic acids to effectively inhibit sporulation was an important finding in the current study, as was the findings of synergistic effects also occurring against parasitic sporulation *in vitro*. These findings were consistent with Nouri (2022), who reported that encapsulated organic acids (mainly lactic and acetic acids) impacted the productivity, survival rate and sporulation percentage. The dosage of organic acids is important, as high doses can decrease feed intake and reduce body weight. (Cruz-Polycarpo et al., 2020) This supports using a low dose of organic acids in the current study.

The current study was the first to investigate the anti-sporulation properties of the combination of citric and formic acids versus a commercial product on chicken *Eimeria* oocysts. Nevertheless, the exact mechanism remains unclear. Further, *in vivo* research is recommended to assess the combination effects on sporozoites, merozoites and intestinal epithelial cells.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by the Science, Research and Innovation Promotion Fund, Thailand Science Research and Innovation (TSRI), through Rajamangala University of Technology Tawan-Ok (RMUTTO) (Grant No. 67A167000004). Ms. Wipa Khiewsalab carried out the molecular detection process and Ms. Natirat Suvajasuwan advised on the morphological approaches.

References

Abbas, R.Z., Munawar, S.H., Manzoor, Z., Iqbal, Z., Khan, M.N., Saleemi, M.K., Zia, M.A. Yousaf, A. 2011. Anticoccidial effects of acetic acid on performance and pathogenic parameters in broiler chickens challenged with *Eimeria tenella*. Pesq. Vet. Bras. 31: 99−103. doi.org/10.1590/S0100-736X2011000200001

Balta, I., Marcu, A., Linton, M., Kelly, C., Stef, L., Pet, I., Ward, P., Pircalabioru, G.G., Chifirius, C., Gundogdu, O., Callaway, T., Corcionivoschi, N. 2021. The in vitro and in vivo anti-virulent effect of organic acid mixtures against *Eimeria tenella* and *Eimeria bovis*. Sci. Rep. 11: 16202. doi.org/10.1038/s41598-021-95459-9

Conway, D.P., McKenzie, M.E. 2007. Poultry coccidiosis: Diagnostic and testing procedures, 3rd ed. Blackwell publishing. Ames, IA, USA.

Cruz-Polycarpo, V.C., Freschi, J.B., Polycarpo, G.V., Araujo, R.G.A.C., Barbieri, A., Sousa, R.L.M. 2020. Isolated and combined organic acids in diets of broiler chickens challenged with *Eimeria acervulina*. Arq. Bras. Med. Vet. Zootec. 72: 1511−1521. doi.org/10.1590/1678-4162-11647

Felici, M., Tugnoli, B., Piva, A., Grilli, E. 2021. In vitro assessment of anticoccidials: Methods and molecules. Animals. 11: 1962. doi.org/10.3390/ani11071962

Gessner, P.K. 1995. Isobolographic analysis of interactions: An update on applications and utility. Toxicol. 105: 161−179. doi.org/10.1016/0300-483x(95)03210-7

Gyorke, A., Pop, L., Cozma, V. 2013. Prevalence and distribution of *Eimeria* species in broiler chicken farms of different capacities. Parasite. 20: 50. doi.org/10.1051/parasite/2013052

Haug, A., Thebo. P., Mattsson, J.G. 2007. A simplified protocol for molecular identification of *Eimeria* species in field samples. Vet Parasitol. 146: 35−45. doi.org/10.1016/j.vetpar.2006.12.015

Kaewthamasorn, M., Charoenvisal, N., Chansiripornchai, N. 2015. Efficacy of salinomycin, robenidine and decoquinate against infection with *Eimeria* species field isolate in a densely populated broiler farm in Thailand. Thai J. Vet. Med. 45: 247−253. doi.org/10.56808/2985-1130.2642

Khan, R.U., Naz, S., Raziq, F., Qudratullah, Q., Khan, N.A., Laudadio, V., Tufarelli, V., Ragni, M. 2022. Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. Environ. Sci. Pollut. Res. Int. 29: 32594–32604. doi.org/10.1007/s11356-022-19241-8

Long, P.L., Joyner, L.P. 1984. Problems in the identification of species of Eimeria. J. Parasotol. 31: 535−541. doi.org/10.1111/j.1550-7408.1984.tb05498.x

Mai, K., Sharman, A.P., Walker, A.R., Katrib, M., Souza, D.D., McConville, J.M., Wallach, G.M., Belli, I.S., Ferguson, J.P.D. Smith, C.N. 2009. Oocyst wall formation and composition in coccidian parasites. Mem. Inst. Oswaldo Cruz. 104: 281−289.doi.org/10.1590/s0074-02762009000200022

Mani-López, E., García, H.S., López-Malo, A. 2012. Organic acids as antimicrobials to control Salmonella in meat and poultry products. Food Res. Int. 45: 713–721. doi.org/10.1016/j. foodres.2011.04.043

Mares, M.M., Al-Quraishy, S., Abdel-Gaber, R., Murshed, M. 2023. Morphological and molecular characterization of *Eimeria spp*. infecting domestic poultry Gallus gallus in Riyadh city, Saudi Arabia. Microorganisms. 11: 795. doi.org/10.3390/microorganisms11030795.

Mathis G.F., Lumpkins B., Cervantes H.M., Fitz-Coy S.H., Jenkins M.C., Jones M.K., Price K.R., Dalloul R.A. 2024. Coccidiosis in poultry: Disease mechanisms, control strategies, and future directions. Poult. Sci. 104663. doi.org/10.1016/j.psj.2024.104663

Mesa-Pineda C., Navarro-Ruiz J., Lopez-Osorio S., Chaparro-Gutierrez J.J., Gomez-Osorio L.M. 2021. Chicken coccidiosis: From the parasite lifecycle to control of the disease. Front Vet Sci. 8: 787653. doi: 10.3389/fvets.2021.787653

Nouri, A. 2022. Anticoccidial and immunogenic effectivity of encapsulated organic acids and anticoccidial drugs in broilers infected with *Eimeria spp*. Sci. Rep. 12: 17060. doi.org/10.1038/s41598-022-20990-2

Ojimelukwe A.E., Emedhem D.E., Agu G.O., Nduka F.O., Abah A.E. 2018. Populations of *Eimeria tenella* express resistance to commonly used anticoccidial drugs in southern Nigeria. Int J Vet Med. 6(2): 192-200. doi: 10.1016/j.ijvsm.2018.06.003

Soulsby, E.J.L. 1986. Helminths, Arthropods and Protozoa of Domesticated Animals, 7th ed. Balliere Tindall. London, UK.

Stephen, B., Rommel, M., Daugschies, A., Haberkorn, A. 1997. Studies of resistance to anticoccidials in *Eimeria* field isolates and pure *Eimeria* strains. Vet Parasitol. 69: 19–29. doi.org/10.1016/s0304-4017(96)01096-5

Thienpont, D., Rochette, F., Vanparijs, O.1979. Diagnosing helminthiasis through coprological examination. Janssen Research Foundation. Beerse, Belgium.

Toschi, A., Rossi, B., Tugnoli, B., Piva, A., Grilli, E. 2020. Nature-Identical compounds and organic acids ameliorate and prevent the damages Induced by an inflammatory challenge in Caco-2 cell culture. Molecules. 25: 4296. doi.org/10.3390/molecules25184296

Williams, R.B. 1997. Laboratory tests of phenolic disinfectants as oocysticides against the chicken coccidium *Eimeria tenella*. Vet. Rec. 141: 447−448. doi.org10.1136/vr.141.17.447

Williams, R.B. 1998. Epidemiological aspects of the use of live anticoccidial vaccines for chickens. Int. J. Parasitol. 28: 1089–1098. doi.org/10.1016/ s0020- 7519(98)00066-6

You, M.J. 2014. Suppression of *Eimeria tenella* sporulation by disinfectants. Korean J. Parasitol. 52: 435−438. doi.org/10.3347/kjp.2014.52.4.435

Zaman, M.A. Iqbal, Z., Abbas, R.Z., Khan, M.N. 2012. Anticoccidial activity of herbal complex in broiler chickens challenged with *Eimeria tenella*. Parasitol. 139: 237−243. doi.org/10.1017/S003118201100182X