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Anticoccidial activities of *Piper betle* L essential oil on *Eimeria tenella* oocysts

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Coccidiosis poses a significant threat to the poultry industry, with synthetic antibiotics and disinfectants being the primary tools for control. This study investigated the potential of *Piper betle* L essential oil (PBEO) as a natural alternative against *Eimeria tenella*, one of the most pathogenic *Eimeria* species affecting poultry. Our findings revealed that PBEO exhibits significant anticoccidial effects through two primary mechanisms: (i) oocysticidal activity by disintegrating oocyst walls and (ii) inhibition of the sporulation process. PBEO demonstrated oocysticidal activities ranging from 8.67 to 95.33% across concentrations from 0.04 to 40%. Notably, at 72 h post-incubation, a 0.04% PBEO concentration significantly reduced the number of sporulated oocysts ($P \leq 0.05$) to 71.67%, showing effects comparable to those of formalin. PBEO reduced 50% of oocyst sporulation (IC_{50}) in the concentration of 1.31% at 72 h. Gas chromatography-mass spectrometry (GC-MS) identified the primary constituents of PBEO, including eugenol, beta-caryophyllene, and other key compounds, collectively constituting 96% of the oil. This research underscores the potential of PBEO as a natural anticoccidial agent and lays the groundwork for further studies aimed at identifying, isolating, and developing active compounds that may specifically target the sporogony process in coccidian parasites.

Keywords Essential oil, Coccidiosis, Ethnopharmacology, Poultry

With the continuous growth of the human population, the demand for animal-based products has significantly increased. Chicken has become a primary source of meat supply, being among the most widely consumed meat products[1]. Among diseases affecting poultry production, avian coccidiosis remains as a significant challenge[2]. Seven *Eimeria* species are known to be causative agents of coccidiosis, i.e. *E. maxima*, *E. necatrix*, *E. acervulina*, *E. praecox*, *E. mitis*, *E. brunetti*, and *E. tenella*[2–5]. The global economic loss attributable to coccidiosis is estimated at approximately US$13 million[6,7], encompassing expenses related to drugs, disinfectants, vaccines, mortality, chick replacement, and high-feed consumption coupled with low feed conversion rates[6–8]. Besides the vaccination strategy and disinfectants as preventives[9,10], the standard curative methods for coccidiosis are antibiotics containing sulfa substances[11]. However, widespread resistance to anticoccidial drugs has raised serious concerns for the poultry and organic industries and results of their residues in meat, eggs, and chicken by-products[12–15].

While *E. necatrix* is identified as the most pathogenic species in Europe and USA[5,16], *E. tenella* is known to be the most prevalent pathogenic species in Southeast Asia[3,5]. *E. tenella* infects cecal epithelial cells as the predilection site in poultry[17]. The short development period of avian coccidia and monoxenous life cycle greatly contribute to the high prevalence of coccidiosis[2]. Coccidiosis infection occurs after the oral uptake of sporulated oocysts. Thereafter, sporocysts are exposed owing to mechanical friction in the gizzard. Sporozoites ($n=8$ per oocyst) are released from the sporocysts under the influence of bile and trypsin and invade intestinal

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mucosal epithelial cells, where schizogony and gametogony to occur[4,5,18]. As disruption to the intestinal epithelium, chickens experience difficulties in digesting food and absorbing nutrients, leading to dehydration, luminal bleeding, lethargy, and a weakened immune system. The chickens also exhibit decreased appetite, become lethargic, and are more susceptible to secondary disease infections. Further, progressive and multiple pathogens co-infections can provoke death in chickens. Clinical manifestations of *E. tenella* infection include bloody diarrhea due to erythrocyte hemorrhage and rupture of the intestinal mucosa during the development of merogony II[2,18,19]. After successful merogony, the gametogony process produces unsporulated oocysts, which are shed in fecal samples, starting the sporogony in the environment[4,5,18]. The unsporulated oocyst consists of a single, undifferentiated cytoplasmic mass protected by a complex, multi-layered wall structure and remains noninfectious until the process is completed[4,20]. Sporogony starts from four haploid sporoblasts enclosed by a shell developing into sporocysts, each containing two sporozoites. Further, the sporont produces a refractile polar body after meiosis. This external sporogony process occurs independently from the host and requires optimal environmental conditions, including adequate aeration, moisture, and a temperature range of 16–23 °C[4,21]. The transmission of coccidiosis to susceptible hosts relies on dispersal of the infectious stage produced after this sporogony process, known as sporulated oocysts[2,4,9].

The oocysts of *Eimeria* sp. exhibit remarkable resistance to environmental conditions and chemical treatments, remaining infectious in the absence of proper disinfection. Their robust walls render them impervious to a range of environmental and chemical challenges, including mechanical, chemical, proteolytic degradation including numerous detergents and disinfectants[20,22]. This resilience enables their survival for extended periods, furthering transmission between hosts. Proper thorough cleaning and disinfection are important for the prevention and management of infections in poultry farms. Chemical-based disinfectants are commonly utilized to assess their efficacy in combating *Eimeria* oocysts. While Virkon S® and TH4®have been recommended for use in poultry farms, particularly in the fight against viral diseases such as highly pathogenic Avian Influenza, Gumboro, and Rabies, none of these compounds effective for coccidia[23,24]. Formalin and sodium hypochlorite, when used in high concentrations, are known to remain effective due to their high reactivity[10,23,25].

Recently, numerous studies have been conducted to investigate natural herbs as an eco-friendly solution for treating coccidiosis[23,26–29]. Herbal plants offer numerous benefits as they are environmentally safety, leave no residues, and are naturally abundant in specific locations[26]. The herb, *P. betel* L., has been studied extensively for its therapeutic properties. *P. betle* L. is easily cultivated 300–1000 m above sea level, especially in soils of the tropical hemisphere with high amounts of organic matter and water[30]. It has pharmacological properties such as analgesic[31], antibacterial[32], anti-mosquito larvae[33], anti-ulcer[34], anti-allergy[34], antioxidant[35], insect repellent[33,36], and anti-diabetic[37]. Despite possessing a diverse array of anti-pathogenic compounds, no studies have been conducted to assess its efficacy against *E. tenella*, a species known to be highly pathogenic to poultry in tropics. To address this research gap, we conducted an in vitro study to evaluate the effectiveness of *P. betle* L. essential oil (PBEO) against *E. tenella* oocysts. Our investigation included gas chromatography-mass spectrometry (GC-MS) analysis to identify the possible active compounds within PBEO. This approach aimed to uncover the potential of PBEO as a natural alternative for controlling *E. tenella* in poultry.

Materials and methods
Ethical approval
The use of experimental animals in this study was approved by the Research Ethics Committee, Ahmad Dahlan University, Indonesia with approval letter number 022206035. Additionally, permission to establish the experiment has been granted on March 15, 2023, by the Animal Science Graduate Committee at Universitas Sebelas Maret to RR, in the letter form of S-3B. Ethical and animal welfare standards were strictly adhered to during experimental procedures involving animals (chicken) and were conducted in accordance with ARRIVE guidelines. All experimental procedures were performed in accordance with the relevant national and institutional guidelines and regulations, including the ethical standards set forth by the Research Ethics Committee of Ahmad Dahlan University, Animal Science Graduate Committee at Universitas Sebelas Maret and in compliance with the Indonesian government regulations on animal research.

Animal
Day-old chicken (DOC) was purchased from "Sahabat Sejati Farm", Central Java, Indonesia. The chicks were vaccinated against Newcastle Disease and Infectious Bronchitis. Before DOC were loaded, the cages were sterilized with Rodalon™ (Medion, Indonesia) and limestone until use. Chicks were placed in individual battery cages with a feces container under the cell. The temperature was constant, and the lighting regimen provided 24 h of continuous light daily. Water and BR Feed (Comfeed, Indonesia) were delivered to the animals without anticoccidial drug. Chickens were reared until the age of 21 days. After reaching 21 days old, each chicken was administrated with Sulfadimethylpyrimidine (Sulfamix®, Medion, Indonesia) as anticoccidial by intramuscular injection for three days. Subsequently, the chickens were raised for a period of one week to eliminate the influence of anticoccidials. When the chickens reached the age of four weeks, a fecal examination was conducted using the flotation method to assess the presence of coccidia followed by an infection challenge using sporulated oocysts. Coccidia-free chicken ($n = 20$) were selected to be infected with pure strains of *E. tenella* oocysts isolated from our previous report[3].

Eimeria tenella infection
Selected chickens were infected orally with 3×10^4 pure strains of sporulated *E. tenella* oocysts[25]. Chicken feces were collected from individual fecal containers on days 4–7 after infection. Then, feces were properly homogenized, filtered using a 100-mesh filter, centrifuged at 4000 revolutions per minute (rpm), and deposited for 10 min. Oocysts were purified by flotation technique using a saturated sugar solution with a ratio of 1:1. The

floatation containers were covered with a square glass plate, allowing the free-floating oocysts to adhere and subsequently be collected by rinsing with water[18,38,39]. Oocysts collected were counted and processed for the assays thereafter.

Gas chromatography–mass spectrometry (GC–MS) assay of *Piper betle* L. essential oil (PBEO)

In this study, PBEO was extracted from the locally sourced *P. betle* L, cultivated in Daerah Istimewa Yogyakarta, Indonesia. Collected leaves were observed and identified by Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada, no. 01244/S.Tb./II/2018. The extraction method was the water-steam distillation implemented in our previous study of *Aedes aegypti*[33]. Briefly, approximately 5 kg of fresh *P. betle* L. leaves were dried for 8 h in a drying cabinet at 50 °C temperature. The dried leaves were steamed with boiling water at a temperature of 100 °C for 4–5 h. The essential oil was produced by passing the vapors through a Clevenger-type apparatus. The resulting essential oil was stored in a sterile glass container, with a total volume of 2.1 ml and golden yellow color.

GC-MS analysis of PBEO was performed at the Advanced Characterization Laboratory, National Research, and Innovation Agency [(Badan Riset dan Inovasi Nasional (BRIN)], at Cibinong, Indonesia. The PBEO was dissolved with dichloromethane to make a 1000 µg/ mL solution and filtered by using a minisart syringe membrane 0.22 µm. The filtrate was injected into a GC-MS instrument Shimadzu GCMS-QP2020NX (Shimadzu, Japan) equipped with an Rtx-5MS column (5% diphenyl: 95% dimethylpolysiloxane), with length of 30 m and diameter of 0.25 mm. The mobile phase consisted of ultra-high purity helium on 30 kPa. The injector temperature was set at 200℃, the ion source at 230℃, and the interphase at 280℃, with a split less injection mode. The oven temperature program was initiated at 60℃ and increased to 150℃ at a rate 10℃/minute and hold for 3 min. The resulting chromatogram and m/z were compared with the National Institute of Standards and Technology (NIST-17) database to identify the detected compounds within the PBEO.

Sporulation inhibition assays

PBEO solution was prepared using Tween 80 and 98% absolute ethanol as solvents, for the formation of smaller micelles[40]. The concentration was adjusted into potassium dichromate 4% as sporulation medium[41]. Potassium dichromate 4% solution served as drug-negative control and formalin as a chemical-based substance (drug-positive control).

Unsporulated *E. tenella*, which were newly obtained from fecal samples, were quantified, and added to diluted substances. For concentration-dependent assays, unsporulated oocysts were incubated in a 1.5 ml microtube with prepared final concentrations of PBEO. Final concentrations of the PBEO and formalin solutions were 0.04%, 0.4%, 4%, 40%, 0.3125%%, 0.625%, 1.25%, 2.5% and 5%. For positive control of chemical substances, formalin was diluted in a 1.5 ml microtube with equal concentrations. The oocysts were observed under light microscopy at 72 h post incubation for microscopic observation. For time-dependent assays, unsporulated oocysts were incubated in 20% PBEO, 20% formalin (drug-positive control), and sporulation medium containing 4% potassium dichromate (drug-negative control). The oocysts sporulation process was evaluated after 6, 12, 18-, 24-, 48-, and 96-h post incubations. All experiments for concentration-dependent and time-dependent assays were performed in triplicate.

The sporulation processes were evaluated based on the following parameters: divided sporocysts, the integrity of the oocyst wall and the number of intact oocysts[25]. The morphologies were evaluated by counting 100 oocysts for each experimental group under a light microscopy[42]. The inhibition of sporulation was determined by comparing percentage of sporulated oocysts between the treated and control groups. The presentation of sporulation was expressed in percent and was calculated using the following formula:

Sporulated oocysts (%) = $\frac{\text{number of sporulated oocysts}}{100}$ x 100%[42].

Scanning electron microscopy

The oocyst samples in 40% PBEO, 40% formalin, and 4% potassium dichromate were processed for scanning electron microscopy (SEM) to visualize morphology on the wall integrity. For SEM preparation, the oocysts from the control and treated groups were washed. The samples were put into a sample container containing absolute ethanol and sent to the Zoology Laboratory, BRIN, at Cibinong, Indonesia for further processing. The sample was prepared according to Goldstein et al. (1992) with a slight modification. Briefly, the oocysts were immersed in cacodylate buffer for 2 h, then agitated with an ultrasonic cleaner for 5 min. The oocysts were then prefixed in 2.5% glutaraldehyde for 2 h. Fixation was conducted in 2% tannic acid for 6 h, and then washed 4 times in cacodylate buffer (5 min each wash). Dehydration was performed by using a serial dilution of alcohol at room temperature. The samples were treated twice with tert-butanol (10 min each) and lyophilized thereafter. Low-speed centrifugation was performed for each solution change during sample preparation. The dried samples were adhered to specimen stubs and coated with aurum using an ion coater. SEM visualization utilized the JSM-IT200 (JEOL, Japan).

Statistical analysis

GraphPad Prism 8.0 (GraphPad Inc., USA) performed statistical analyses and graphical visualizations. The results were presented as mean values ± standard deviation and considered statistically significant when probability values (P-values) were less than 0.05 ($P \leq 0.05$).

Results

Inhibition of *Piper betle* L. essential oil (PBEO) to *Eimeria tenella* sporulation

The study revealed that in the control incubations with 4% K2Cr2O7, 90% of *E. tenella* oocysts exhibited complete sporulation after a 72-h incubation period and morphologically both inner and outer wall layers were

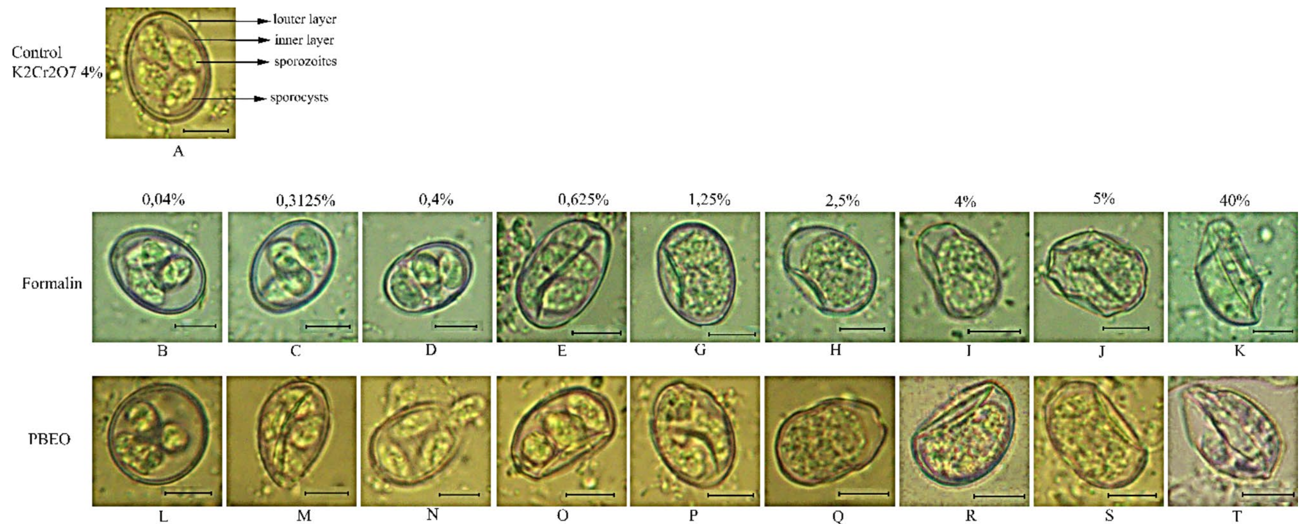

Figure. 1. Morphology of *Eimeria tenella* oocysts treated with various concentrations of *Piper bettle* L essential oil (PBEO) and formalin after incubations for 72 h. (**A**) 4% $K_2Cr_2O_7$ control in 72 h of incubation time. (**B-K**) Oocysts in 0.04%, 0.3125%, 0.4%, 0.625%, 1.25%, 2.5%, 4%, 5%, and 40% formalin. (**L-T**) Oocysts in 0.04%, 0.3125%, 0.4%, 0.625%, 1.25%, 2.5%, 4%, 5%, and 40% PBEO. Scale bars represented 10 µm.

PBEO concentration	Sporulated oocysts	*P* compared to untreated group
0.04%	71.67%	0.00038*
0.3125%	61.67%	0.00008*
0.4%	60.00%	0.00048*
0.625%	46.33%	0.00004*
1.25%	38.33%	0.00000*
2.5%	36.33%	0.00000*
4%	35.00%	0.00004*
5%	15.00%	0.00000*
40%	10.00%	0.00000*

Table 1. Sporulation assay of *Eimeria tenella* oocyst in *Piper bettle* L essential oil (PBEO) with different concentrations range at 72 h.

intact (Fig. 1A; Table 1). Oocysts incubated in formalin at 0.04% (Fig. 1B) and 0.3125% (Fig. 1C) displayed incomplete sporulation, yet their oocyst walls remained intact. Incomplete sporulation also occurred in oocysts in formalin at 0.4% and 0.625%, accompanied by deformation of the oocyst walls (Fig. 1D, E). Severe deformations of the oocyst walls were observed in formalin incubations at 1.25% (Fig. 1G), 2.5% (Fig. 1H), 4% (Fig. 1I), 5% (Fig. 1J), and 40% (Fig. 1K), and oocysts were unable to sporulate completely. Oocysts were able to sporulate in incubations with 0.04% PBEO, and oocyst walls remained intact (Fig. 1L). However, deformations in oocyst walls occurred in the later concentrations of PBEO, specifically 0.3125 – 0.4% (Fig. 1M-N). Most oocysts incubated with 0.625%, 1.25%, 2.5%, 4%, 5%, and 40% PBEO experienced deformation of the oocyst walls (Fig. 1O-T) and were unable to sporulate completely.

PBEO in a 20% concentration significantly inhibited sporulation starting from the first 6 h of incubation time (Table 2). In the control experiments involving 4% K2Cr2O7, the sporulation rates of *E. tenella* oocysts were 13.03%, 20.87%, 41.87%, 64.37%, 80.13%, and 100% after 6, 12, 18, 24, 48, and 96 h of incubation, respectively. Incubation in 20% formalin and PBEO significantly reduced the ability of *E. tenella* to sporulate ($P \le 0.05$) by 96 h compared to the control (Fig. 2A). The percentage of oocysts able to sporulate was also significantly lower in a series of formalin and PBEO dilutions (w/v; 0.04%, 0.4%, 4%, 40%, 0.3125%, 0.625%, 1.25%, 2.5%, and 5%) ($P \le 0.05$) compared to the control (Fig. 2B). The IC50 of formalin and PBEO in this study were 1.03% and 1.31% at 96 h, respectively (Fig. 2C).

Oocysticidal activities of *Piper betle* L. essential oil (PBEO) to *Eimeria tenella* oocyst

Disintegrated oocysts occurred during incubation in a concentration- and time-dependent manner in both PBEO and formalin solutions (Fig. 3A, B). In the control incubations, which utilized a 4% solution of K2Cr2O7, no disintegration of *E. tenella* oocysts was observed after 96 h (Fig. 4.A-G). Oocysts treated in formalin solutions (Fig. 4H) exhibited significant disintegration rates ranging from 63.33 to 100% after 6, 12, 18, 24, 48, and 96 h

Incubation time	Sporulated oocysts	*P* compared to untreated group
6 h	1.67%	0.0146*
12 h	5.69%	0.0028*
18 h	9.93%	0.0198*
24 h	10.73%	0.0000*
48 h	25.10%	0.0037*
96 h	37.90%	0.0010*

Table 2. Sporulation assay of *Eimeria tenella* oocyst in the *Piper bettle* L essential oil (PBEO) at 20% concentration in different incubation time.

of incubations, respectively (Fig. 4.I-N). The disintegration of oocysts incubated in various dilutions (w/v; 40, 5, 4, 2.5, 1.25, 0.625, 0.4, 0.3125, and 0.04%) of PBEO were significantly higher than control after 96 h, with a corresponding $P \leq 0.05$ (Fig. 2). As the concentration of the substance increased, the rate of oocyst disintegration also increased (Table 3). Intact unsporulated-oocysts (Fig. 4O) exhibited disintegration when incubated in 20% PBEO, resulting in varying rates ranging from 61.67 to 93.33% after being incubated for 6, 12, 18, 24, 48, and 96 h, respectively (Table 4; Fig. 4P-U). The total number of disintegrated oocysts incubated in 20% PBEO was significantly different from the control incubations (4% K2Cr2O7) after 6, 12, 18, 24, 48, and 96 h ($P \leq 0.05$), but it was not significantly different from the 20% formalin-treated groups ($P \geq 0.05$).

Scanning electron microscopy

SEM analysis of *E. tenella* oocysts in the control medium revealed that they were ovoid in shape and featured smooth surfaces (Figs. 1A and 5A). Following incubation in 40% formalin for 72 h, the oocyst walls displayed a remarkable degree of collapse (Figs. 1K and 5B). When incubated in 40% PBEO for 72 h, the oocyst ruptured, exposing the disintegrated oocyst wall (Fig. 1T, 5C).

Gas chromatography assay of Piper betle L. essential oil (PBEO)

The GC-MS from the PBEO profile (Supplementary Material S1, S2) comprised of 50 peaks, 49 were successfully identified and only one was unidentified. The analysis from GC revealed various molecular components, such as hydrocarbons, bicyclic compounds, aromatic compounds, and oxygenated compounds. Hydrocarbons were found to include alkanes, such as 2,4-dimethylheptane, 3,7-dimethyldecane, 5-methyltetradecane, 2,6,11-trimethyldodecane, 4,6-dimethyldodecane, n-heptadecane, n-heneicosane, and eicosane. Additionally, alkenes, including 7,11-dimethyl-3-methylene-(e)-1,6,10-dodecatriene, and cycloalkanes, such as 1,7-dimethyl-7-(4-methyl-3-pentenyl)-tricyclo [2.2.1.0(2,6)] heptane and 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-(s)-cyclohexen, were identified. The bicyclic compounds identified included 1-methyl-4-(1-methylethyl)-benzene, estragole, and 4-(2-propenyl)-phenol, eugenol, 4-allylphenyl acetate, 3-allyl-6-methoxyphenyl acetate, and 4-allyl-1,2-diacetoxybenzene, along with terpenoids and related compounds such as gamma-terpinene, linalol, (-)-terpinen-4-ol, l-alpha-terpineol, copaene, and humulene. The alcohols group found included eucalyptol, 1-methyl-4-(1-methylethyl)-benzene, linalol, (-)-terpinen-4-ol, l-alpha-terpineol, 1-isopropyl-4-methylenebicyclo [3.1.0] hexane, and 2-isopropyl-5-methyl-1-heptanol.

In our analysis, the predominant compounds identified were eugenol (7.96%), 1,2,3,5,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-naphthalene (6.47%), decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-naphthalene (6.16%), 4-allylphenyl acetate (5.67%), 3-allyl-6-methoxyphenyl acetate (5.47%), 4,11,11-trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene (5.21%), n-heptadecane (4.97%), 4-(2-propenyl)-phenol (3.63%, humulene (3.76%) and (-)-terpinen-4-ol (3.58%).

Discussion

The study of herbal plants for their anti-pathogenic effects, particularly by extracts and essential oils, has been widely recognized[43]. Pine bark extract (*Pinus radiata*) has demonstrated inhibition of *E. tenella* sporulation, up to 80% at 48 h of incubation, when utilized at a concentration of 0.1%[44]. Water-soluble green tea extract has proven to be effective in reducing oocyst sporulation of *E. tenella*, *E. acervulina*, and *E. maxima*. Green tea extract treatment with 5% and 10% concentrations reduced *E. tenella* sporulation by 41.14% and 64.2%, respectively, after 48 h of incubation[45]. A methanol extract of 1% *Rosmarinus officinalis* reduced *E. tenella* oocysts sporulation up to 50% after 48 h of incubation. The 3% *Citrus aurantium* ethanolic extract able to inhibit sporulation after 48 h of incubation[25]. The effectiveness of these herbal extracts was determined by two parameters: (i) concentration and (ii) incubation time. Our study demonstrated that PBEO significantly inhibited *E. tenella* sporulation in a concentration- and time-dependent manner. Inhibition of sporulation was observed with a concentration as low as 0.04% at 72 h and as the concentration of PBEO increased, the total number of sporulated oocysts decreased accordingly. PBEO exhibited inhibition of the sporulation process as early as 6 h post incubation time, in a 20% concentration. Inhibition of sporulation is an important mechanism for controlling coccidiosis, as unsporulated oocysts remain noninfectious, consequently reducing coccidian parasite dispersal and disrupting the life cycle. While some chemical disinfectants have the capacity to damage oocysts, they often lack the ability to inhibit *Eimeria* sporulation[46]. The poultry industry recognizes the importance of disinfection and sanitary depopulation of broiler flocks between check-outs and check-ins to reduce infection pressure and control coccidiosis. However, most products available with disinfection action on oocysts are caustic or toxic, making

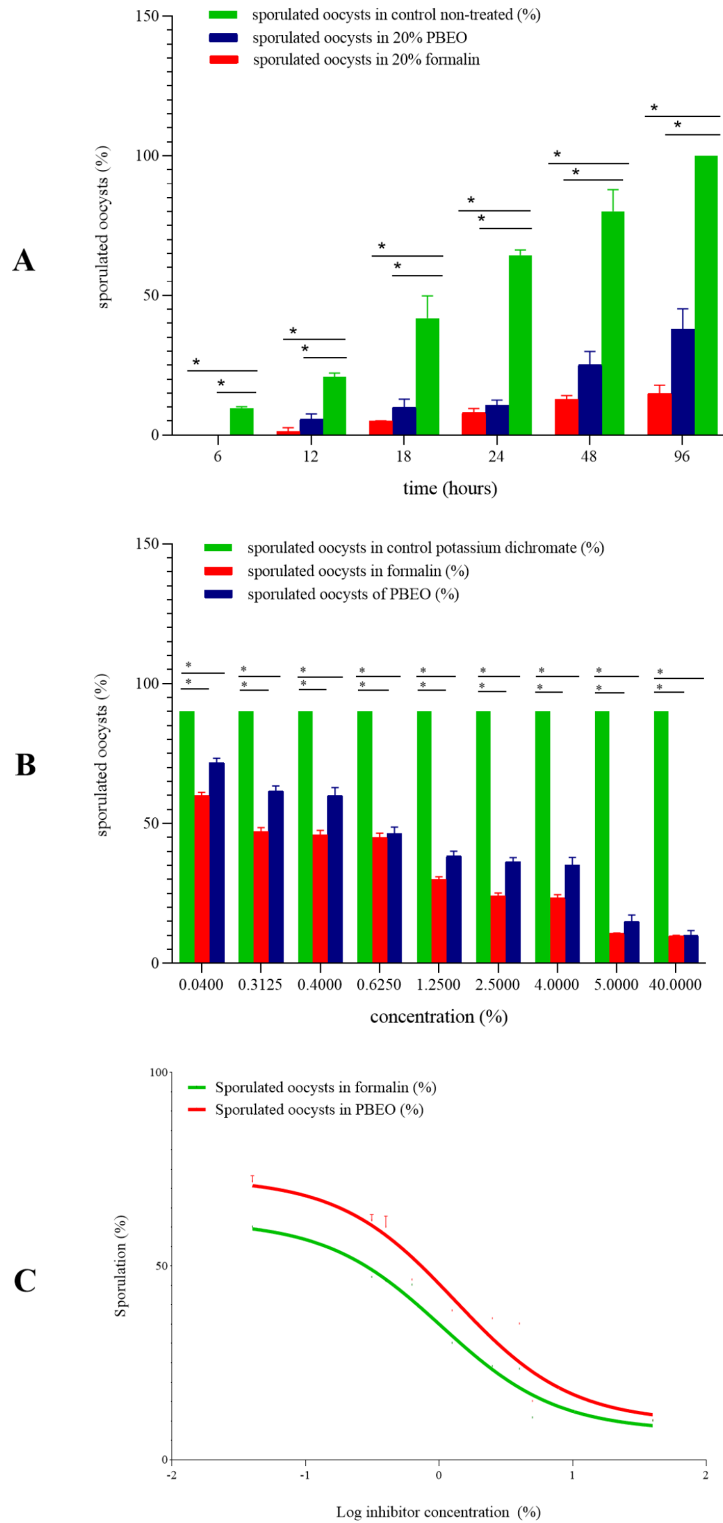

Figure 2. Formalin and *Piper bettle* L essential oil (PBEO) effects on *Eimeria tenella* sporulation process. **(A)** Sporulated *E. tenella* oocysts (%) treated with 20% PBEO, 20% formalin, and 4% $K_2Cr_2O_7$ control in a time-dependent manner. **(B)** Sporulated *E. tenella* oocysts (%) treated with different concentrations of PBEO, formalin, and 4% $K_2Cr_2O_7$ control. **(C)** Dose-response curve of the sporulation assays by PBEO and formalin incubations at 72 h. * significant ($P \leq 0.05$).

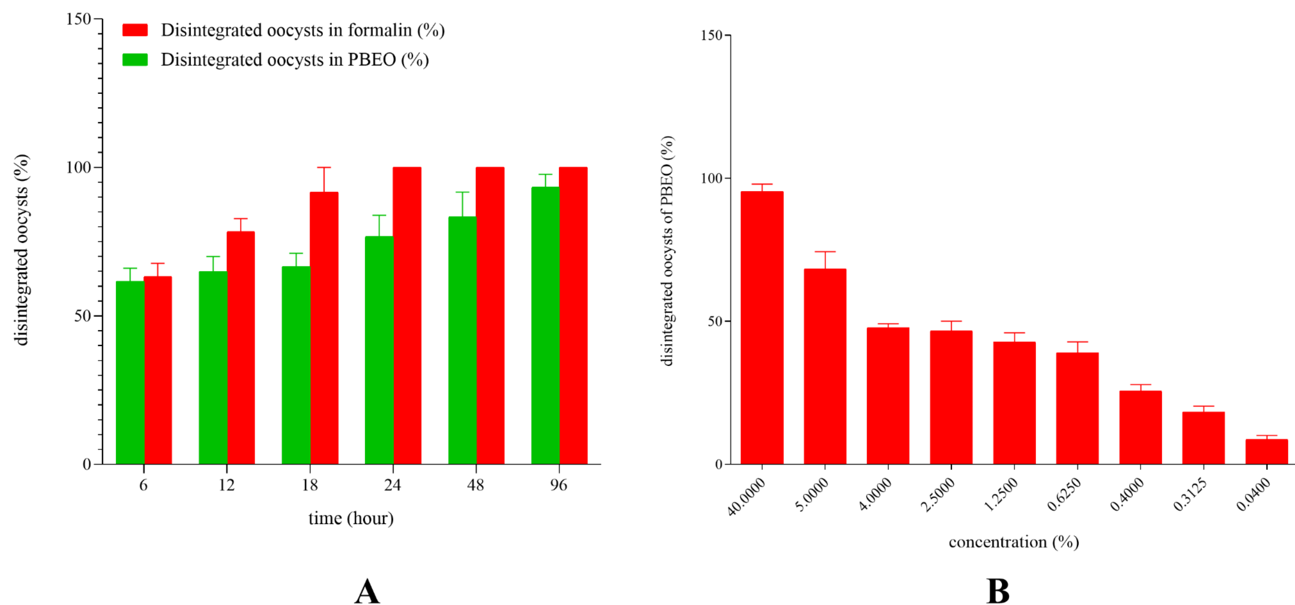

Figure 3. Oocysticidal activities of formalin and *Piper bettle* L essential oil (PBEO) to *Eimeria tenella* oocysts. **(A)** Disintegrated *E. tenella* oocysts treated with 20% PBEO, 20% and formalin in different incubation times. **(B)** Number of disintegrated oocysts (%) incubated in different concentration ranges.

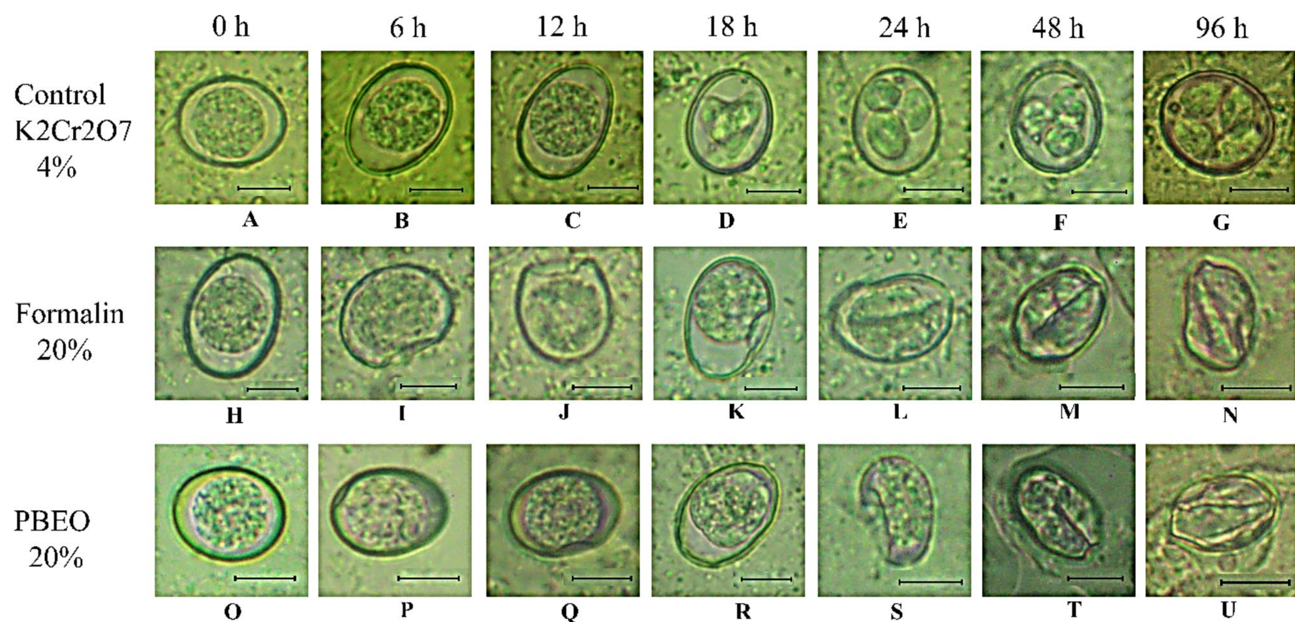

Figure 4. Morphology of *Eimeria tenella* oocysts treated with 20% formalin, 20% *Piper bettle* L essential oil (PBEO), and 4% $K_2Cr_2O_7$ at different incubation times. **(A-G)** Oocysts were treated with 4% K2Cr2O7 with 0, 6, 12, 18-, 24-, 48-, and 96-h incubation times. **(H-N)** Oocysts were treated with formalin with 0, 6, 12, 18-, 24-, 48-, and 96-h incubations. **(O-U)** oocysts treated with PBEO from 0, 6, 12, 18-, 24-, 48-, and 96-h incubations. Scale bars represented 10 µm.

them unsuitable for use in poultry installations by safety issue[47]. Therefore, PBEO's ability to effectively inhibit sporulation at low concentrations is of such significance in our study. The inhibition of sporulation is attributed to the phytochemical compounds present in certain herbs[48]. *R. officinalis*, for instance, contains polyphenol compounds that impede oxygen ingress and restrain the activity of mannitol dehydrogenase enzyme[25]. Similarly, *P. radiata* possesses 35% tannin, which has been proposed to inactivate sporulation, as seen in helminth eggs[44,49,50]. Green tea extract, contains polyphenols and tannins[45]. Earlier studies suggested that terpenoids in herbal plants can obstruct the sporulation of *E. tenella*. Terpenoids can penetrate the oocyst and bind with carbohydrates and lipids, thus reducing the oxygen levels and the activity of endogenous enzymes[51].

PBEO concentration	Disintegrated oocysts	*P* compared to untreated group
0.04%	8.67%	0.00398*
0.3125%	18.33%	0.00083*
0.4%	25.67%	0.00030*
0.625%	39.00%	0.00050*
1.25%	42.67%	0.00020*
2.5%	46.67%	0.00015*
4%	47.67%	0.00000*
5%	68.33%	0.00034*
40%	95.33%	0.00000*

Table 3. Oocysticidal assay of *Eimeria tenella* in the *Piper bettle* L essential oil (PBEO) at different concentrations range at 72 h.

Incubation time	Disintegrated oocysts	*P* compared to untreated group
6 h	61.67%	0.0001*
12 h	65.00%	0.0002*
18 h	66.67%	0.0001*
24 h	76.67%	0.0004*
48 h	83.33%	0.0005*
96 h	93.33%	0.00002*

Table 4. Oocysticidal assay of *Eimeria tenella* oocyst in the *Piper bettle* L essential oil (PBEO) at 20% concentration in different incubation time.

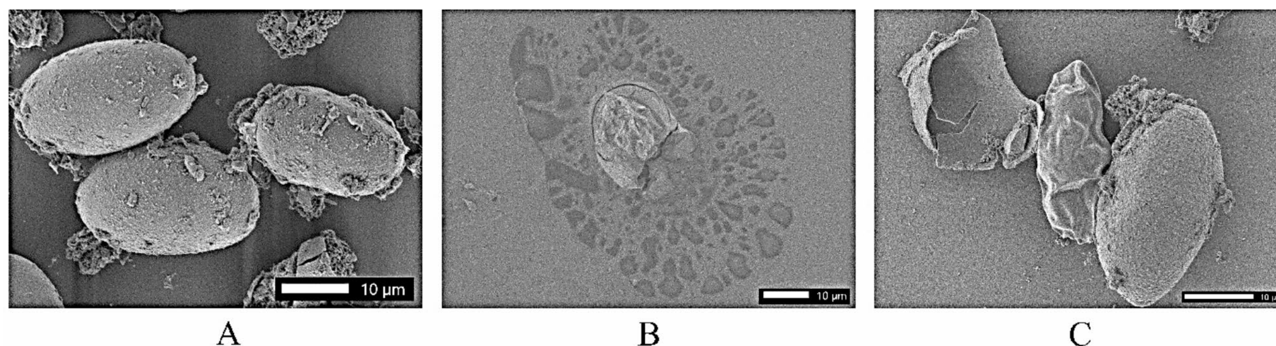

Figure 5. (**A**)*Eimeria tenella* oocysts from the control group showed an ovoid shape with intact walls and smooth surfaces. (**B**) Oocyst in 40% formalin showed total wall disintegration after 72 h incubation. (**C**) Oocyst-wall treated with *Piper bettle* L essential oil (PBEO) 40% was remarkably ruptured after incubation for 72 h.

Botanical herbs may contain hundreds or even thousands of individual constituents at varying levels of abundance[52]. Identifying bioactive compounds within a complex herbal solution can be challenging but detecting metabolite concentrations can help pinpoint the components responsible for their effects in the early stages of research[53,54]. Our GC analysis revealed the presence of 50 metabolites of our extracted PBEO, among which polyphenol eugenol, was the most prevalent constituent. Eugenol has reportedly improving the cecal microbial structure, decreasing inflammatory reactions, strengthening immune function and demonstrating anticoccidial in vivo[55]. Dietary supplementation of clove oil, which contains eugenol as a main component, has shown protective effects on chickens' performance and health status against coccidiosis[56]. However, no studies have examined eugenol's role in sporogony, the stage that occurs independently in the environment (in vitro). During sporogony, *Eimeria* relies solely on its own energy reserves, without a host system, and mannitol is believed to be a critical energy source during this stage. About 90% of the mannitol in unsporulated oocysts is consumed within the first 15 h of sporulation, with levels decreasing as sporulation completes, typically over 35 h[57]. The various constituents within herb essential oil can inhibit single enzyme or multiple enzymes in a pathway through the combined action of multiple compounds, leading to a synergistic effect that exceeds the sum of their individual actions[58,59]. This synergy has the potential to reduce the required dosage of each compound, thereby maximizing therapeutic outcomes while minimizing side effects. Advocates of botanical

medicine often emphasize the synergistic interactions between components, resulting in a combined impact that may surpasse the efficacy of each part alone. Therefore, following the identification of a crude extract with promising pharmacological activity, bioactivity-guided fractionation is a critical next step for further research[53].

Instead of merely inhibiting the sporogony process, PBEO exhibited oocysticidal effects by collapsing the oocyst and sporocyst walls at specific concentrations. The essential oil penetrated and perforated the oocyst wall, a process potentially facilitated by its terpenoids and phenolic components[60]. Once inside, PBEO affected the deeper layer, the sporocyst, leading to damage and abnormalities in the cytoplasmic contents. Essential oils are also known to create a hypertonic environment for oocysts[61]. The oocysticidal activity of PBEO was comparable to that of formalin[25,62], which was used as the drug-positive control in our experiment and demonstrated significant oocysticidal effects. This observation aligns with previous studies on formalin's mechanism of action, which involves disrupting oocysts by penetrating their interior and reacting with biological compounds[62]. Formalin achieves this through chemical reactions with primary amide and amino groups of proteins, thereby inactivating pathogenic microorganisms[47,63,64]. However, while formalin is an effective disinfectant for poultry houses and litter, it is toxic to both animals and humans[65,66]. In contrast, PBEO not only demonstrates effective oocysticidal properties but also does so with non-toxic characteristics, making it a promising alternative.

Other in vitro studies are essential to verify the ability of sporozoites, through artificial excystation and treatment with PBEO, to invade susceptible host cells and complete the merogony stages. PBEO contains natural polyphenolic components that are known to inhibit the invasion of *E. tenella* sporozoites after oral ingestion of the oocyst[67]. Additionally, saponins, which can react with cholesterol in the sporozoite membrane, prevent host cell invasion[68], suggesting that PBEO could have a multi-faceted mechanism of action against coccidiosis. Conducting cell culture experiments will provide valuable insights into the cellular mechanisms involved, allowing for a more in-depth understanding of how PBEO affects the life cycle of *E. tenella*.

In vivo studies have already shown that PBEO has potential as a safe and effective treatment, particularly when considering the recommended dosages of eugenol-containing essential oils[55]. Further testing PBEO on chicks during challenge infections, followed by an analysis of total oocyst production, can provide information on *E. tenella*'s ability to invade host cells when treated with PBEO prior to administration. For practical applications, research on various formulation strategies is required. For example, dietary supplementation of clove essential oil has been shown to improve broiler performance and mitigate coccidia-related damage to gut integrity and meat quality[69]. Encapsulation of essential oils in nano-emulsions presents an effective alternative to antibiotics, increasing surface area and ensuring a more consistent delivery through smaller droplet sizes[70]. This highlights the need for further research into the formulation strategies that could enhance the efficacy of PBEO, such as its use as a feed additive in chickens to control coccidiosis[71,72].

To fully explore the potential applications of PBEO, we propose further investigation into the isolation, purification, and toxicity testing of its active substances. The isolation of active components from PBEO can aid in the standardization and consistent efficacy of these alternatives. Such research would not only optimize the therapeutic use of PBEO but also ensure its safety and effectiveness as a sustainable alternative to traditional treatments for coccidiosis in poultry. Moreover, evaluating the long-term impacts of PBEO on poultry health and productivity could help establish it as a reliable component of integrated disease management strategies in commercial poultry production.

Conclusion

Our research demonstrates that PBEO exhibits both oocysticidal and sporulation-inhibitory activities, positioning it as a promising alternative for controlling coccidiosis during the environmental stage of poultry production. Notably, a PBEO concentration of 0.04% significantly inhibited sporulation, with an IC50 of 1.31% observed against oocyst sporulation after 72 h of incubation. Oocysts exposed to a 20% PBEO concentration showed disintegration rates ranging from 61.67 to 93.33% after 6 to 96 h of incubation. Future research can be performed on bioactivity-guided fractionation to identify the most effective compound against avian coccidiosis and advance the chemical characterization of these compounds. This will further elucidate the therapeutic potential of PBEO and contribute to the development of more effective and sustainable strategies for coccidiosis control in poultry.

Data availability

All data supporting the findings of this study are available in the paper and its Supplementary Information section. GC-MS results and analysis are provided in Supplementary Tables 1 and 2.

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Author contributions
R.R. contributes to writing original drafts, investigation, methodology, visualization, and formal analysis.P.H.H. contributes to reviews and editing drafts, conceptualization, project administration, supervision, funding acquisition, validation, data curation, and resources.H.A.N contribute to resources, investigation, and methodology-.T.R.N contributes to reviews and editing drafts, validation, and data curation.S.W. contributes to editing drafts, validation, and data curation.P.R.F. contributes to visualization, investigation, and formal analysis.R.L.R.A. contributes to visualization, investigation, and formal analysis.T.M.C., S.K., A.H.W., and M.M. contribute to reviews and editing drafts, validation, data curation, and methodology.

Competing interests
The authors declare no competing interests.

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