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Research Article
Changes in Sporulation, Packed Cell Volume, Malondialdehyde Level, Fecal Oocyst Count and Histopathology of *Eimeria tenella*-infected Broilers Treated with Pineapple (*Ananas comosus*) Crude Extracts

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Abstract

Objective: This experiment was conducted to study the effects of pineapple (*Ananas comosus*) peel and core extracts for controlling *in vitro* and *in vivo* chicken cecal coccidiosis. **Materials and Methods:** *In vitro*, 5,000 unsporulated oocysts per well with three replicates were divided into 4 groups: incubated in distilled water, toltrazuril, pineapple peel and core extract, respectively, at 25°C for 48 h. The number of sporulated oocysts was counted using McMaster chamber method. *In vivo*, 100 heads of one-day-old male broilers were divided into 5 groups: control, infected with *E. tenella* oocysts, infected and treated with toltrazuril, infected and treated with pineapple peel extract and infected and treated with pineapple core extract. All infected groups were inoculated with 20,000 sporulated *E. tenella* oocysts at day 21 of the experiment. After 7 days of infection, all of the chicks were euthanized and the blood, feces and cecum were collected for analysis. **Results:** *In vitro* results showed that the number of sporulated *E. tenella* oocysts in the pineapple peel and core extract groups decreased significantly ($p<0.05$). Correspondingly, histopathological study of the cecum showed that infection of *E. tenella* was greatly decreased in these groups. Surprisingly, pineapple extract groups had a significant increase in malondialdehyde level ($p<0.05$), which was correlated with increased inflammatory infiltration in cecal tissue of these groups. **Conclusion:** Pineapple crude extract inhibited sporulation and decreased the chance of *E. tenella* infection, but may be toxic by inducing cecal tissue inflammation.

Key words: Chicken cecal coccidiosis, pineapple, *Eimeria tenella*, inflammation, cecum

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Avian coccidiosis is one of the most economically important diseases of the poultry industry, caused by apicomplexan parasites belonging to the genus *Eimeria*. There are seven species in this genus that affect chickens, among which *E. tenella* is one of the most pathogenic[1]. Infection with *E. tenella* is followed by cecal lesions (petechiae, thickening, ecchymosis, accumulation of blood and caseous necrotic material in the cecum), accompanied with bloody diarrhea[2]. Intensive poultry production systems depend on chemoprophylaxis with anticoccidial drugs to combat infection. Anticoccidial drugs have been used for over 60 years and their extensive use has led to the development of drug-resistant *Eimeria* spp. strains[3-5] which are responsible for subclinical coccidiosis and economic losses due to poor weight gain and high food consumption. Based on the health and economic damage mentioned above, there is increasing interest in the development of alternative strategies for disease control and prevention in poultry[6], including new molecules and vaccines. As part of this effort, studies on the inhibitory effects of natural products on *Eimeria* were recently published[7-9]. This study was aimed to investigate the possible anticoccidial effects of pineapple (*Ananas comosus*) peel and core on packed cell volume, malondialdehyde level, fecal oocyst count and lesion score of *Eimeria tenella*-infected broilers, as well as an *in vitro* sporulation inhibition assay.

MATERIALS AND METHODS

Animals and management: One hundred 1-day-old broiler chicks were divided into 5 groups: control, infected with *E. tenella* oocysts, infected and treated with the anticoccidial drug toltrazuril, infected and treated with pineapple peel extract and infected and treated with pineapple core extract. Diet formulas contained no anticoccidial feed additives. Chicks were offered feed and water *ad libitum*. The experiment was reviewed and approved by the Institutional Animal Care and Use Committee, Mahasarakham University (approval number: 0001/2016).

Parasites: *Eimeria tenella* was obtained from Associate Professor Somboon Sangmaneedate, Faculty of Veterinary Medicine, Khon Kaen University, Thailand. Oocysts were propagated, isolated and sporulated using standard procedures[10] at the Parasitology Laboratory, Faculty of Veterinary Sciences, Mahasarakham University, Thailand. The oocysts were induced to the sporulation stage in an aqueous solution of potassium dichromate 2.5% (w/v) for 48 h, then each chick was infected at day 21 of the experiment by a suspension of 2×10^4 sporulated oocysts in a volume of 1 mL.

Pineapple crude extraction: Pineapple extraction followed the method of Ketnawa *et al.*[11]. Pineapples (*Ananas comosus*) were purchased from a local market. The peel and core were removed and cut into small pieces, then blended for 3 min with cold distilled water (1:1) using a juice blender (Tefal, Jakarta, Indonesia). The material was filtered with a muslin cloth and centrifuged (Hettich, Kirchlengern, Germany) for 20 min at 10,000×g and 4°C. The supernatants were then collected as pineapple peel and core extracts and kept at 4°C in a refrigerator until used in each week of the experiment.

Pineapple extract and toltrazuril administration: Pineapple peel and core extracts (freshly prepared every week) were diluted in distilled water at 1% concentration and administered in drinking water every day until the end of the experiment. For the anticoccidial drug group, toltrazuril (7 mg kg^{-1} b.wt.) was administered in drinking water on days 18 and 19 of the experiment, otherwise, the animals were given normal drinking water.

Sporulation inhibition assay: An *in vitro* assay was performed to evaluate the effects of pineapple peel and core extracts on the sporulation of *E. tenella* oocysts. In this study, 5,000 unsporulated oocysts per well with 3 replicates were divided into 4 groups. The control group was incubated in distilled water; the test groups were incubated in toltrazuril, pineapple peel and pineapple core extract, respectively, at 25°C for 48 h. At the end of incubation, the oocysts were washed twice in tap water. Then the number of sporulated oocysts was counted using McMaster chamber method.

Packed cell volume: Blood samples were collected from the wing vein of chicks using a 3 mL disposable syringe and directly transferred into labeled test tubes containing anticoagulant (EDTA). Samples were then placed in microhematocrit tubes and packed cell volume was determined by a hematocrit centrifuge.

Malondialdehyde level: Lipid peroxidation in chick blood was determined using 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid, followed by heating in a boiling water bath for 30 min. Thiobarbituric acid reactive

substances were determined by the absorbance at 532 nm and expressed as malondialdehyde equivalents formed.

Oocyst output per gram of feces: Fecal samples from infected broilers in each group were pooled at day 7 post-infection. The oocysts were processed by concentrating in saturated sodium chloride solution and counted using a McMaster chamber under a light microscope (Olympus, Tokyo, Japan).

Lesion scoring: On day 7 post-infection, all chicks were sacrificed by cervical dislocation. The intestine was immediately exposed by a ventral midline incision to macroscopically examine lesions. This evaluation was based on a lesion scoring system[12], which provided a numerical ranking of gross lesions using a discrete 5-point scale (0 = No lesions, 1 = Mild lesions, 2 = Moderate lesions, 3 = Severe lesions and 4 = Extremely severe lesions or death).

Histopathological changes: Cecal tissue was fixed in 10% formalin, then processed according to routine histological techniques and embedded in paraffin. Each sample block was sectioned at a thickness of 4 μm using a rotary microtome (Leica® Germany). Cecal tissue sections were stained with hematoxylin–eosin and histopathological changes observed under a light microscope.

DNA extraction and Polymerase Chain Reaction (PCR): The DNA was extracted using a DNA extraction kit (GF-1, Vivantis, Oceanside, C A, USA), then the PCR protocol was performed for confirmation of *E. tenella* infection in poultry. Primers for *E. tenella* was designed as follows: ETF-AATTTAGTCCATCGCAACCCT and ETR-CGAGCGCTCTGCATACGACA[13]. The PCR mixture contained 0.5 μM of each primer, 2.5 mM $MgCl_2$, 0.5 mM dNTP and 1 μL of extracted DNA, with nuclease-free water added to a final volume of 10 μL. Amplification was performed using a ProFlex PCR system thermal cycler (Applied Biosystems, Foster City, CA, USA) at initial denaturation of 94°C for 3 min, followed by 35 amplification cycles (94°C for 30 sec, 63°C for 30 sec and 72°C for 90 sec) and a final extension at 72°C for 15 min. The amplification of specific PCR products was checked by gel electrophoresis in 2% agarose gel stained with 0.5 μg mL^{-1} ethidium bromide.

Statistical analysis: Data were analyzed using one-way analysis of variance. All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 21.0 for windows (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p<0.05$.

RESULTS

Sporulation inhibition assay: The number of sporulated oocysts in the pineapple peel extract groups was significantly less ($p<0.05$) compared with the control and toltrazuril groups. Pineapple core extract had a significantly stronger effect ($p<0.05$) on sporulation inhibition than pineapple peel extract, while pineapple peel extract and toltrazuril had similar effects (Table 1).

Packed cell volume: Packed cell volume in all experimental groups was at a normal level, with no significant difference (Table 2).

Malondialdehyde level: Malondialdehyde (MDA) was used to assess lipid peroxidation in chick blood. The MDA level of both plant extract groups increased significantly ($p<0.05$), compared with the untreated groups. Notably, the MDA level of the pineapple peel group was two times greater than the control group (Fig. 1).

Oocyst output per gram of feces: There was no difference in oocyst output per gram of feces among all experimental groups. The control group showed no oocyst output in feces (Table 2).

Table 1: Effects of pineapple extracts on *E. tenella* oocyst sporulation *in vitro*

Groups	Number of sporulated oocysts
Control	2,540.44±146.32^{c}
Toltrazuril	2,052.62±193.37^{b}
Pineapple peel	1,913.38±173.01^{b}
Pineapple core	1,417.87±37.58^{a}

Values are Mean±SD, $^{a-c}$Values followed by different superscripts are significantly different ($p<0.05$)

Table 2: Effects of pineapple extracts on packed cell volume, oocysts per gram of feces and lesion scoring of the cecum in experimental groups

Groups	Packed cell volume (%)	Oocysts per gram of feces (×10^5)	Lesion score
Con	25.00±0.00^{a}	00.00±00.00^{b}	0.00±0.00^{a}
Et	30.50±3.42^{a}	80.33±0.87^{a}	2.20±0.84^{b}
EtD	25.67±2.52^{a}	69.45±0.66^{a}	1.60±0.55^{b}
EtP	25.33±2.52^{a}	71.13±0.80^{a}	2.20±0.45^{b}
EtC	25.33±1.53^{a}	60.24±0.65^{a}	2.20±0.45^{b}

Values are Mean±SD. Con: Control, Et: Infected with *E. tenella*, EtD: Infected with *E. tenella* and treated with toltrazuril, EtP: Infected with *E. tenella* and treated with pineapple peel extract, EtC: Infected with *E. tenella* and treated with pineapple core extract, $^{a-b}$Values followed by different superscripts letters are significantly different ($p<0.05$)

Lesion scoring: Most of the lesions in all infected chicks were bloody, showing thickening and petechial hemorrhage of the cecal wall. In some of the chicks, a cecal core was found in the lumen of the cecum. There was no difference in the average cecal lesion scores of all treated groups (p>0.05), the lowest score was in the toltrazuril-treated group, which was correlated with oocyst output per gram of feces (Table 2).

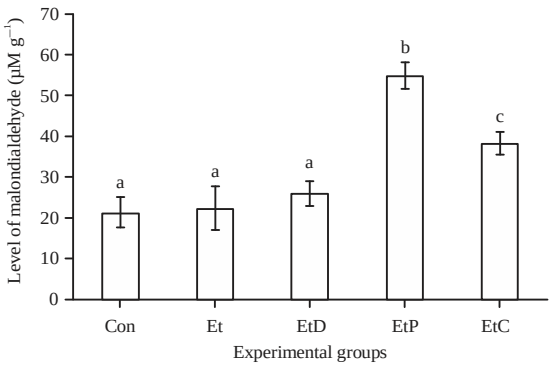

Fig. 1: Malondialdehyde levels of experimental groups.
Values are Mean±SD. Con: Control, Et: Infected with *E. tenella*, EtD: Infected with *E. tenella* and treated with toltrazuril, EtP: Infected with *E. tenella* and treated with pineapple peel extract, EtC: Infected with *E. tenella* and treated with pineapple core extract, $^{a-c}$ Significantly different (p<0.05)

Histopathological changes: Histopathological changes and the number of parasites in tissue were observed in all groups under a light microscope. The cecum in the control group displayed a normal structure. The main histopathological changes in all infected groups were in the cecal wall, the changes included villous atrophy, inflammatory infiltration, hemorrhage, epithelial loss and tissue infection with different stages of *E. tenella*. *Eimeria tenella* infection severely damaged the cecum, this damage was slightly alleviated in the toltrazuril-treated group (Fig. 2a, b). Interestingly, the number of tissue parasites at various stages obviously decreased in both pineapple peel and core extract-treated groups (Fig. 2c, d).

DNA extraction and polymerase chain reaction (PCR): To confirm infection by *E. tenella*, DNA was extracted from the infection site and PCR was performed using specific primers for *E. tenella*. Gel electrophoresis of PCR amplification products showed a positive specific band at the 275 base pair site, except in the control group where this band was not found (Fig. 3).

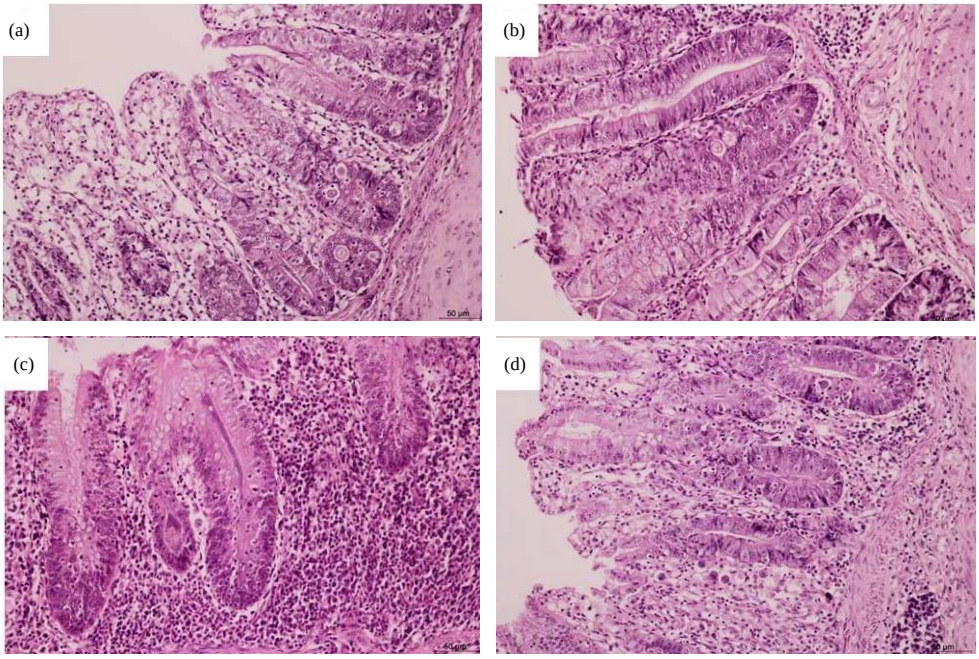

Fig. 2(a-d): Histopathological results of infected groups (hematoxylin and eosin staining): A: Infected with *E. tenella*, B: Infected with *E. tenella* and treated with toltrazuril, C: Infected with *E. tenella* and treated with pineapple peel extract, D: infected with *E. tenella* and treated with pineapple core extract

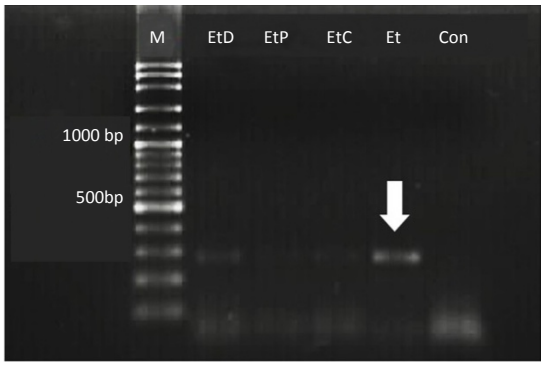

Fig. 3: Results from gel electrophoresis of specific PCR products.
Con: Control, Et: Infected with *E. tenella*, EtD: Infected with *E. tenella* and treated with toltrazuril, EtP: Infected with *E. tenella* and treated with pineapple peel extract, EtC: Infected with *E. tenella* and treated with pineapple core extract, M: Marker, bp: Base pair, White arrow: Specific band

DISCUSSION

The anticoccidial activity of pineapple peel and core extracts was investigated in this study. The results of the *in vitro* study showed that pineapple extract acts like toltrazuril by inhibiting sporulation of *E. tenella* oocysts, as evidenced by a significant lowering in the number of sporulated *E. tenella* oocysts after incubation with these plant extracts for 48 h. Enzymes from pineapple extracts are in close contact with oocysts and their activity could inhibit sporulation[14]. Pineapple is an important source of the cysteine protease enzyme, bromelain, which can be extracted from many parts of the pineapple, especially the stem[15,16]. Bromelain has proteolytic activity that can break the peptide bonds of the protein structure[16,17]. The proteolytic properties of this enzyme seem to have the ability to impair sporulation development of *E. tenella* oocysts. However, the inhibition mechanism is unknown and requires further study.

The effects of bromelain have been researched in previous studies, including: Inhibition of platelet aggregation[18,19], anti-inflammatory[15,19-22] and anti-tumor[20-23] properties, induction of wound healing and skin decrement[19,24,25] and anthelmintic properties and activity against cow ticks[16,26-30]. In the present study, broilers treated with pineapple peel and core extracts showed no difference in packed cell volume, oocyst output per gram of feces and lesion score compared with the untreated groups. The main histopathological changes in *E. tenella* infection were villous atrophy, inflammatory infiltration, hemorrhage, epithelial loss and tissue infection with different stages of *E. tenella*, which were observed in all infected groups. *E. tenella* infection caused severe damage to the chicken cecum, which was slightly alleviated in the toltrazuril-treated group, in accordance with previous studies[31-33]. Surprisingly, increased inflammatory cell infiltration in cecal tissue was observed in the pineapple peel and core extract groups; this result was related to the increase in malondialdehyde levels in the chick blood of both groups. Lipid peroxidation occurs due to damage in the intestine, leading to oxidative stress and inflammation of the intestinal mucosa[34,35]. Increased inflammatory cell infiltration in cecal tissue resulted in severely elevated malondialdehyde levels, indicating that pineapple extract may be an irritant substance on the intestinal mucosa in cases of chicken cecal coccidiosis. The induction of inflammatory cells in this study disagreed with several previous reports which indicated that bromelain has an anti-inflammatory effect[36-38]. However, Hale *et al*.[38] hypothesized that proteolytically active bromelain may have a mild irritant effect on the mucosa and this may have been the cause of inflammatory cell induction in this study.

CONCLUSION

In conclusion, the results from this study showed that pineapple crude extract inhibited sporulation and decreased infectivity of *E. tenella*, indicating that this plant could be used as an anticoccidial substance for controlling *E. tenella* infection. However, proteolytic enzymes from pineapple extract may be toxic to the cecal mucosa in cases of *E. tenella* infection and induce cecal tissue inflammation. Therefore, strategic control of *E. tenella* infection requires finding a substance with high anticoccidial ability as well as antioxidant properties to reduce the degree of intestinal lipid peroxidation and neutralize reactive oxygen species. Finally, the mechanisms of pineapple extract for inhibiting sporulation and reducing infectivity of *E. tenella* oocysts for the control of chicken cecal coccidiosis should be investigated in future studies.

SIGNIFICANCE STATEMENT

This study discovered that the effects of pineapple peel and core extract can be beneficial for the inhibition of sporulation and decreased infectivity of *E. tenella* oocysts in broilers. Thus, pineapple crude extract, which is a natural compound, can be used to control infection by cecal coccidia in chickens and may also affect other chicken parasites. However, the results of this study showed elevation of malondialdehyde levels and induction of cecal tissue

inflammation by pineapple crude extract. Therefore, the mechanisms of pineapple extract for inhibiting sporulation and reducing infectivity of *E. tenella* oocysts for the control of chicken cecal coccidiosis should be clarified in further studies.

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