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Effect of the Natural Extract of Juglans Mandshuria Epicarp on Broiler Coccidiosis Disease

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Abstract

Coccidiosis is a protozoal disease caused by Eimeria that significantly impacts the global poultry industry. In this study, in vitro and in vivo experiments were conducted to evaluate the anticoccidial effect of Juglans mandshurica epicarp aqueous extract as an alternative treatment against coccidiosis in broiler chickens. In the in vitro experiment, unsporulated Eimeria tenella oocysts were exposed to various concentrations of the aqueous extract. After 48 hours of incubation, the degree of inhibition of sporulation and morphed oocysts were examined. For the in vivo experiment, seven groups of two-week-old broiler chickens were divided into A1-A5, positive control (PC), and negative control (NC) groups. The A1-A4 groups received different doses of the aqueous extract calculated based on toxicity tests, while group A5 was fed diets mixed with salinomycin. Groups A1-A5 were infected with E. tenella, while PC was infected but unmedicated, and NC was uninfected and unmedicated. Results showed that the aqueous extract inhibited the sporulation of E. tenella oocysts in vitro in a concentration-dependent manner. All experimental groups fed with the extract exhibited significantly higher weight gain, particularly those receiving 5 mL/kg BW. Moreover, the groups receiving doses of 5 and 7 mL/kg BW showed significant differences in bloody Diarrhea and cecal lesion scores compared to the PC group. The anticoccidial index (ACI) value for these groups was above 160, indicating high efficacy similar to the group fed with mixed salinomycin. In conclusion, the study suggests that J. mandshurica epicarp aqueous extract could be a safe and effective alternative treatment for coccidiosis in broilers. This could provide a new approach to control coccidiosis in the global poultry industry.

Introduction

Coccidiosis is one of the main parasitic diseases caused by protozoan Eimeria in poultry and is recognized as a serious parasitic disease worldwide (Kostadinović *et al.*, 2015). Seven coccidiosis species have been identified in chicken, among which Eimeria tenella, Eimeria brunetti, and Eimeria necatrix are known to be highly pathogenic (Hafez, 2008). Coccidiosis is a major parasitic disease affecting poultry and results in severe economic loss due to severe reductions in feed utilization and body weight gain (Kostadinović *et al.*, 2015).

Coccidiosis control relies mainly on chemicals, which costs much money. The continuous emergence of antibiotic-resistant Eimeria has raised concerns about the safety of anticoccidial medicines and their potential negative impact on human, animal and environmental health (Primož and Suzana, 2012; Nilsson *et al.*, 2012). In this context, the use of anticoccidial chemicals for commercial poultry production is prohibited in many countries and new countermeasures are urgently required along with public distrust of meat using chemical medicines (Giannenas *et al.*, 2003; Applegate *et al.*, 2010; Peek

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and Landman, 2011; Bozkurt *et al.,* 2012). It has been strongly advocated that synthetic medicines should be opposed to natural additives (Alaeldein *et al.,* 2018; Rashed and Alaeldein, 2019; Saleh *et al.,* 2020). The use of plant medicament as one of the natural additives is a new idea since it has several advantages including low cost, safety, low residue or no residue in broilers and many other benefits (Kostadinović *et al.,* 2015; Mahmood *et al.,* 2020).

Juglans mandshuria Maxim, also called Manchurian walnut or Onigurumi, is a perennial deciduous broad-leaf tree that is fast-growing in northeastern China and Korean Peninsula (Wei-Ning *et al.,* 2010; Wen-Ting *et al.,* 2016). The bark, branches, epicarp of immature fruits, leaves, roots, and stems have high therapeutic effects and have thus been extensively used to cure a variety of diseases in the past (Yan *et al.,* 2019). For instance, the epicarp of immature fruits has been demonstrated to be against cancer, dermatosis, diarrhea, dysentery, gastric ulcer, leukopenia, and uterine prolapse (Qing *et al.,* 2010; Arvind *et al.,* 2011; Yuanyuan *et al.,* 2015; Yuwei *et al.,* 2016; Jin-Hai *et al.,* 2018; Yuan-Yuan *et al.,* 2019). The significant anti-inflammatory activity has been reported for extracts of J. mandshurica in several inflammatory models (Xiaohui *et al.,* 2015; Fei *et al.,* 2021). In mice, the treatment with the juglone in the extracts of the immature exocarps of J. mandshurica significantly decreased the levels of TNF-α, IL-1β, and IL-6, proving that it suppressed the inflammatory response through inhibition of the TLR4/NF-κB signaling pathway (Xiaohui *et al.,* 2015).

Juglone (5-hydroxy-1, 4-naphthoquinone), a type of naphthoquinone, is a phenolic compound that exhibits allelopathic properties. It has remarkable antibacterial activity against Gram-positive, Gram-negative bacteria, yeast and fungi (Saling *et al.,* 2011; Qin *et al.,* 2019; Fei *et al.,* 2021).

However, to the best of our knowledge, no studies have been published on the use of J. mandshurica epicarp as an anticoccidial agent. In addition, since J. mandshurica epicarp has been regarded as a discarded part not used for food, its beneficial use in the poultry industry has not yet been explored.

In this study, we have assumed that natural plant extracts can be used as alternatives to synthetic coccidiosis drugs. Therefore, we tested the anticoccidial effect of the aqueous extract of J. mandshurica epicarp.

Materials and Methods

All the animal experiments in our paper were performed by the guidelines of the Institutional Animal Care and Use Committee of DPR of Korea and approved by the Ethics Committee of the Institute of veterinary medicine of the Academy of Agricultural Researchers. All efforts were made to minimize suffering.

Preparation of Eimeria oocyst samples

A single species of E. tenella oocyst was isolated from infected chicken caecum by saltwater float selection and subjected to specific modifications. Unsporulated oocysts were obtained from the caecal contents of chickens 7 days after infection. The oocysts in saturated saline solution were centrifuged for 10 min at 1000g at 4℃, and the supernatant containing the oocysts was transferred to a 1L measuring cylinder filled with distilled water. The cylinder was left overnight at 4 ℃ and then the upper solution was removed not to disturb the pellets, leaving the final volume of 100 mL. Under the abovementioned conditions, the remaining solution (100mL) was further centrifuged to remove the supernatant, and 10 mL of the solution containing oocysts was obtained. Oocysts size was calculated from samples of eighty oocysts isolated from the chicken caeca. The morphology and quantity of oocysts were analyzed under the microscope and finally confirmed by PCR (Fernandez *et al.,* 2003). The oocysts were extracted with a DNA extraction kit (Takara, Japan), and PCR was performed with a Taq DNA polymerases kit (Takara, Japan). The PCR conditions are as follows; an initial denaturation at 94℃ for 3 min, 30 cycles of 45 sec at 94 ℃, 30 sec at 60℃, and 1 min at 72 ℃ for annealing, with a final extension step at 72 ℃ for 5 min. The amplification products were separated on 1.5% agarose gel followed by ethidium bromide staining and visualized under UV light.

To enhance sporulation, the oocysts were put in incubator glass dishes containing 2.5% potassium dichromate solution, which was then aspirated and stirred on the occasion. Daily examination of the dishes proceeded until 90–95% sporulation. The dishes were washed using phosphate buffer solution (PBS, pH=7.2) 3 times before use and diluted to the desired concentration of 1 mL solution.

Preparation of plant extracts and medicine feeding

J. mandshurica epicarp was collected in the mountains around Pyongyang city, DPR of Korea, between August and September. The epicarp of J. mandshurica was dried at 37 ℃ for 48 hours and extracted with water (volume ratio of 1:10) at 37 ℃ for 2 days. Table 1 shows representative active ingredients of J. mandshurica epicarp extract.

The chickens were fed a medicated mixture of feed and medication, where the quantity of medication was calculated based on their body weight. Following the medicated feed, they were given their regular feed and water. The medication was administered during the first feeding of the day for seven consecutive days (Giannenas *et al.,* 2014).

Table 1. The composition of the active ingredients of the extract

No	Chemical constituents	Extracts	Parts
1	Juglone	Water	Green walnut husks
2	2-Hydroxy-1,4-naphthoquinone	Water	Green walnut husks
3	Phenolics	Water	Green walnut husks
4	Tetralones	Water	Green walnut husks
5	Triterpenoids	Water	Green walnut husks
6	Flavonoids	Water	Green walnut husks
7	Phenylpropanoids	Water	Green walnut husks
8	Coumarins	Water	Green walnut husks
9	Steroids	Water	Green walnut husks

In vitro examination procedure

Non-sporulated E. tenella oocysts were exposed to the aqueous extract of different concentrations (0.01, 0.1, 1, 10, 30, 60, 100%) of J. mandshurica epicarp. After incubation at room temperature (26-29℃) for 48 hours, the number of sporulated, non-sporulated, and deformed oocysts was recorded as a percentage. The NC group was exposed to distilled water, and all the experiments were repeated three times.

In vivo examination procedure

A total of two-week-old Cobb chicks (n = 350) (Institute of veterinary medicine of the Academy of Agriculture, Pyongyang, DPR of Korea) were acclimated for one week before the experiment. The test groups consisted of 5 groups (A1-A5, n=10 per group) and the chickens were orally administered with 4×104sporulated oocysts (Table 2).

Table 2. In vivo experimental design[1]

Groups	Chickens	Applied medicines	Dosage mL/kg BW	Days of medicine application
NC	10			
PC	10			
A1	10	The aqueousextract of J. mandshurica epicarp	1	7
A2	10	The aqueousextract of J. mandshurica epicarp	3	7
A3	10	The aqueousextract of J. mandshurica epicarp	5	7
A4	10	The aqueousextract of J. mandshurica epicarp	7	7
A5	10	Salinomycin	66 mg/kg	7

[1]Five replicates were performed for each group of the experiment.
PC: positive control (infected without medication); NC: negative control (uninfected without medication).

Table 3. Composition and nutritional composition of standard diet[1]

Ingredient	Amount, %
Corn	58.2
Soybean meal	28.4
Corn gluten meal	8.4
Corn oil	2.0
Dicalcium phosphate	1.4
Salt	0.3
DL-Methionine	0.2
Lysine	0.1
Vitamin and trace element premix[2]	1.0
Calculated nutrient composition	Amount
ME, kcal/kg	2,830
Crude Protein, %	19.03
Methionine+Cysteine, %	0.9
Methionine, %	0.47
Lysine, %	1.05
Calcium, %	0.95
Available phosphorus, %	0.43
Total phosphorus, %	0.60

[1]Experimental chicks were fed a diet based on the breeder's nutrient recommendations for the Cobb 700 breeder (Cobb-Vantress. 2020)

[2]Minimum guarantee analysis of vitamin and trace element premix: Vit A 10000 IU/kg; Vit D3 1500 IU/kg; Vit E 5 mg/kg, Vit K3 1 mg/kg; Vit B1 0.5 mg/kg; Vit B2 2 mg/kg; Vit B5 (pantothenic acid) 5 mg/kg; Vit B6 5 mg/kg; Vit B12 0.2 mg/kg; Vit PP 10 mg/kg; B9 (folic acid) 0.5 mg/kg; biotin 1 mg/kg; choline chloride 500mg; Fe 60 mg; Zn 60 mg; Mn 60 mg; Cu 5.6 mg; I 0.5 mg; Co 0.3 mg.

The medicine doses of the A1-A4 groups were calculated at 1, 3, 5, and 7 mL/kg BW respectively. The medicine was applied daily from the beginning of oral oocysts administration. The A5 group was fed diets containing salinomycin (Institute of veterinary medicine of the Academy of Agriculture, Pyongyang,

Medicine toxicity test

The toxicity test of J. mandshurica epicarp can be divided into acute toxicity test and chronic toxicity test. For the acute toxicity test, mice were divided into five groups (at doses of 4, 5, 6, 7, and 8 g Kg-1, respectively) with eight mice in each group. The concentrate of epicarp aqueous extraction was dissolved in a reasonable amount of saline and then administered by intraperitoneal injection. After injection, the number of deaths was recorded for 14 days and LD50 was calculated using the Bliss method. For the chronic toxicity test, two-week-old Cobb chicks were divided into the control and test groups (30 chicks in each group), and the number of deaths, appetite loss, depression index, and body weight change were investigated while feeding the medicine at a therapeutic dose (aqueous extraction of 6ml/kg body weight) for one month.

DPR of Korea) 66mg/kg, while the PC group was infected and without medication, and the NC group was uninfected and without medication. Chickens were reared in wire-floor cages. Feed and water are free to eat and the basic rations are guaranteed by the instructions (Table 3).

Assessment of anticoccidial activity

The anticoccidial activity of the aqueous extract of J. mandshurica epicarp was assessed by bloody diarrhea, relative body weight gain(BWG), survival rate, oocyst output/g excreta, and lesion scores. The anticoccidial index was calculated using Dexing *et al.* (2011) method with the following formula:

ACI (anticoccidial index) = (relative ratio of BWG + survival rate) - (lesion scores + oocyst value)

According to ACI values, the anticoccidial effects were determined as follows; ACI >180 was considered excellent, 180-160 was considered marked, 160-140 was considered moderate, 140-120 was considered slight, and ACI <120 was considered inactive (Yasuhiro *et al.*, 1977).

On the 4th–7th days after infection, the chickens were examined for bloody diarrhea by enumerating bloody excreta twice daily. The degree of bloody diarrhea is the average value of blood excreta described by Abbas *et al.* (2010), which can be divided into 0-3 categories according to the blood content of 0, 33, 33-66, and 66-99% in the fecal. Feces from each group were collected between the 4th and 7th days after infection and the oocysts were counted by a hemocytometer according to Holdsworth *et al.* (2004). One gram of each well-mixed feces sample was soaked in 10 mL of water, and 10μL of suspension was taken to calculate the oocysts under the optical microscope. The results have been expressed as oocysts/g output. The oocyst value and oocyst reduction rate were calculated using a method described by Lan *et al.* (2016), Li-yun *et al.* (2021), and Min *et al.* (2019).

$$\text{ROP (relative oocyst production)\%} = \frac{\text{Oocysts per g output of every group}}{\text{Oocysts per g output of the PC group}} \times 100$$

When the ROP is 0-1%, the oocyst value is 0; when the ROP is 1-25%, the oocyst value is 5; when the ROP is 26-50%, the oocyst value is 10; when the ROP is 51-75%, the oocyst value was 20; when the ROP was 76-100%, the oocyst value was 40.

$$\text{Oocysts reduction rate(\%)} = \frac{\text{OPG of PC group} - \text{OPG of experimental group}}{\text{OPG of PC group}} \times 100$$

$$\text{Survival rate(\%)} = \frac{\text{Number of surviving chickens}}{\text{initial number of chickens}} \times 100$$

The relative weight gain rate was calculated by measuring the bodyweight of each group from the beginning date of infection to the final date of infection and subtracting the initial BW from the final BW.

$$\text{Relative ratio BWG} = \frac{\text{Average BWG in each group}}{\text{Average BWG of NC group}} \times 100$$

On the seventh day of infection, 5 chickens of each group were slaughtered, and the cecum of each bird was removed and observed. Lesion scores were marked from 0 to 4 depending on the severity of the cecum.

Lesion value= mean lesion score×10.

The degree of the lesion was determined using the method described by Johnson and Reid (1970). After the initial examination of the appearance of the lesion, the chicken cecum was further cut open to observe the internal lesions of the intestine. According to the severity of chicken cecal lesions, the lesion score was divided into five grades.

Statistical Analysis

All the data were analyzed by SPSS 20.0 software. Significance of the differences in BWG and oocyst discharge of the NC, PC, A1-5 groups were statistically analyzed by one-way ANOVA and recorded as Mean and SEM. In addition, the nonparametric Kruskal-Wallis H test was used to compare the bloody diarrhea score and lesion value among the NC, PC, A1-5 groups. And Dunn's multiple comparison tests were used to analyze statistical

differences between the NC, PC, A1-5 groups. The significancy level was set at $P < 0.05$.

Results

Morphometric analysis of Eimeria species

Based on morphology, the length and the width of oocysts were determined as 21.3 ± 1.44 μM and 18.2±1.68 μM respectively, and the shape index was 1.17±1.08. The results showed that the isolated oocysts were E. tenella. Finally, the oocysts were identified by PCR and the result showed a definite band at 539bp (Figure 1).

Acute and chronic toxicity tests

In the acute toxicity test, LD50 of the aqueous extract of J. mandshurica epicarp was 5.76 g/kg, and its confidence limit was 5.39-6.11g/Kg. The results of chronic toxicity tests demonstrated that there was no difference in the number of deaths, loss of appetite, and depression when 6mL of the aqueous extract of J. mandshurica epicarp (/kg body weight) was given to healthy 2-week-old Cobb chicks every day for one month. However, in terms of weight change, the test groups were slightly higher than the control groups, but there was no significant difference. The results revealed that the extract from the epicarp of J. mandshurica had no negative effect on the growth of chicks.

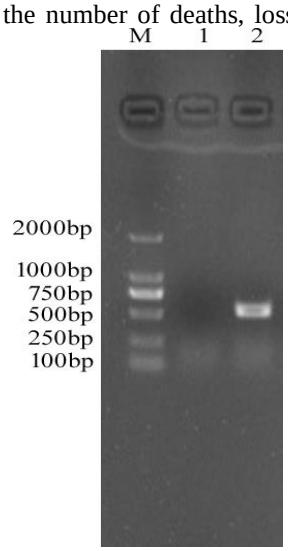

Figure 1. Agarose gel electrophoresis of PCR products using DNA samples of E. tenella (lane 2) and\ a control with no starting DNA (lane 1). Molecular size markers (2000DNA Marker) in base pairs are indicated on the left.

Sporulation inhibition test

The sporulation inhibition effect of the aqueous extract of J. mandshurica epicarp was evaluated by treating isolated E. tenella oocyst with various concentrations. In this experiment, the sporulation rate of oocysts was calculated by recording the number of sporulated, non-sporulated, and deformed oocysts at different concentrations of the aqueous extracts (Figure 2).

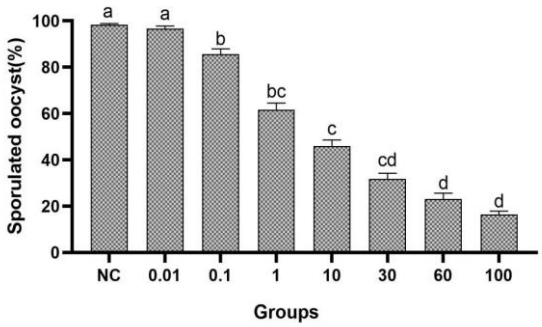

Figure 2. Effect of J. mandshuria extract on sporulation of E. tenella. E. tenella showed sporulation differences in water and different concentration of the aqueous extract of J. mandshurica epicarp. Water was used as a negative control (NC). The mean for three replicates is shown, a–d Different letters indicate statistically significant differences ($P < 0.05$).

As shown in Figure 2, at the concentration of 100, 60, 30, 10, and 1% of aqueous extract, the sporulation rate was 16.3, 23.0, 31.7, 46.0, 61.7%, respectively, compared to the water control group (98%), displaying significant sporulation inhibition effect ($P < 0.05$). Very low concentrations of aqueous extract (0.01% and 0.1%) had no significant sporulation inhibition activity. After being incubated with different concentrations of the aqueous extract for 48 hours, more deformed oocysts were detected (Figure 3).

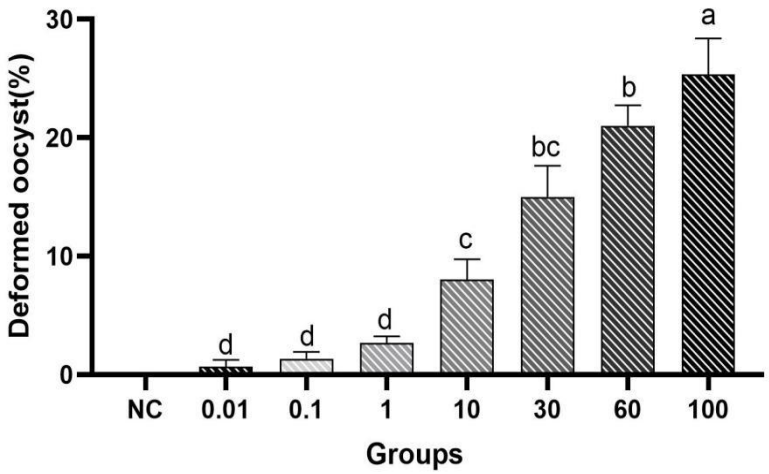

Figure 3. Effect of J. mandshuria extract on deformation of E. tenella oocysts. Water was used as a negative control (NC). The mean for three replicates are shown, a–d Different letters indicate statistically significant differences (P < 0.05).

As shown in Figure 3, the aqueous extract of J. mandshurica epicarp had a great effect on oocyst deformation. The inhibition effect was especially significant when the concentration of the aqueous extract was 10, 30, 60, and 100% ($P < 0.05$).

Effect on relative body weight gain rate

The relative body weight gain rates of chicks infected with E. tenella were measured for different doses of aqueous extract (Table. 4 and Figure 4).

Table 4. Comparison of relative body weight gain, bloody diarrhea, lesion value, oocyst value, and ACI in the different chicken groups

Group	Survival rate (%)	Relative weight gain rate (%)	Bloody diarrhea (Mean)	Lesion value (Mean)	Oocyst value	ACI (Mean)
NC	100.00	100.00^{a}	0.00	0.00	0.00	200.00
PC	80.00	67.40^{d}	3.0^{a}	35^{a}	40.00	72.40
A1	100.00	74.34^{cd}	2.3^{b}	28^{b}	20.00	126.34
A2	100.00	83.01^{c}	1.6^{c}	19^{c}	10.00	154.01
A3	100.00	87.99^{bc}	0.7^{d}	17^{d}	10.00	160.99
A4	100.00	87.40^{bc}	0.6^{d}	17^{d}	10.00	160.40
A5	100.00	90.78^{b}	0.4^{d}	16^{d}	10.00	164.78
SEM	0.84	1.28	0.12	1.22	1.42	2.73
P-Value	<0.0522	<0.038	<0.0269	<0.0285	<0.0313	<0.0116

A1-A4: groups applied with aqueous extract of J. mandshurica epicarp; A1: 1mL aqueous extract/kg bodyweight; A2: 3mL aqueous extract/kg bodyweight; A3: 5 mL aqueous extract/kg bodyweight; A4: 7 mL aqueous extract/kg bodyweight; A5: 66mg salinomycin/kg in feed; PC: positive control (infected without medication); NC: negative control (uninfected without medication); ACI: anticoccidial index; a–d Different letters indicate statistically significant differences ($P < 0.05$).

As shown in Figure 4, the relative body weight gain in the experimental groups was significantly different from that of the control group infected with oocysts but did not receive the medicines ($P < 0.05$). Also, among the experimental groups fed with aqueous extract, the relative body weight gain of group A3 fed with 5 mL/kg BW was relatively high.

Oocysts per gram of feces (OPG)

No oocysts appeared in the feces within 3 days after oocysts inoculation, and there was a difference in the average excretion of oocysts from 4th to 7th days (Table 5).

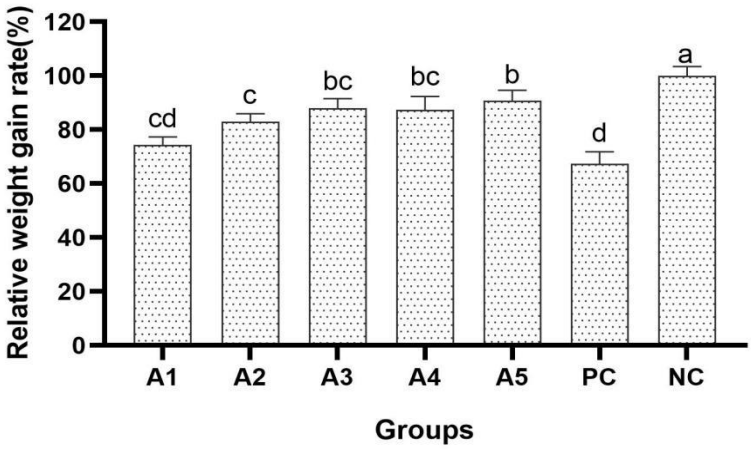

Figure 4. Relative weight gain of chicks infected with E. tenella. A1-A4: groups applied with aqueous extract of J. mandshurica epicarp; A1: 1mL aqueous extract/kg bodyweight; A2: 3mL aqueous extract/kg bodyweight; A3: 5mL aqueous extract/kg bodyweight; A4: 7mL aqueous extract/kg bodyweight; A5: 66mg salinomycin/kg in feed; PC: positive control (infected without medication); NC: negative control (uninfected without medication). a–d Different letters indicate statistically significant differences ($P < 0.05$).

Table 5. Comparison of mean OPG output calculated in the feces of different groups and inhibition rate

Group	OPG (Mean×105)	Inhibition rate (%)
NC	0.00	100.00[a]
PC	31.62[a]	0.00[e]
A1	19.80[b]	37.38[d]
A2	12.98[c]	58.95[c]
A3	10.74[d]	66.03[b]
A4	9.72[d]	69.26[b]
A5	8.29[d]	73.78[b]
SEM	1.14	3.61
P-Value	<0.034	<0.012

A1-A4: groups applied with aqueous extract of J. mandshurica epicarp; A1: 1mL aqueous extract/kg bodyweight; A2: 3mL aqueous extract/kg bodyweight; A3: 5 mL aqueous extract/kg bodyweight; A4: 7 mL aqueous extract/kg bodyweight; A5: 66mg salinomycin/kg in feed; PC: positive control (infected without medication); NC: negative control (uninfected without medication); OPG: oocysts in per gram feces; a–e Different letters indicate statistically significant differences ($P < 0.05$).

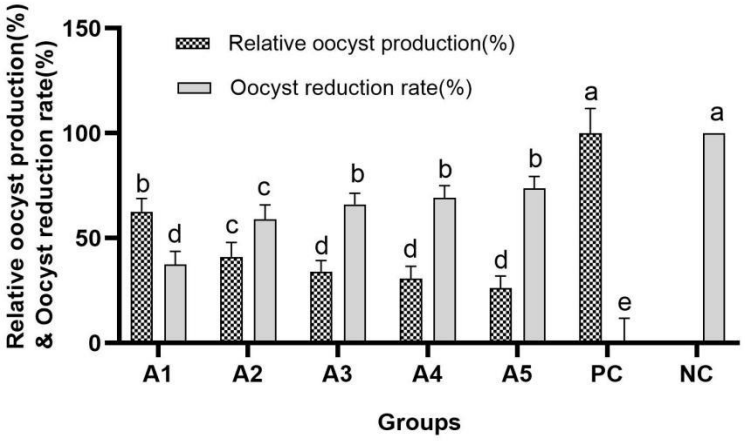

Figure 5. Effect of J. mandshuria extract on oocysts production and reduction rate of E. tenella oocysts after oral inoculation. A1-A4: groups applied with aqueous extract of J. mandshurica epicarp; A1: 1mL aqueous extract/kg bodyweight; A2: 3mL aqueous extract/kg bodyweight; A3: 5mL aqueous extract/kg bodyweight; A4: 7mL aqueous extract/kg bodyweight; A5: 66mg salinomycin/kg in feed; PC: positive control (infected without medication); NC: negative control (uninfected without medication). a–e Different letters indicate statistically significant differences ($P < 0.05$).

A1-A4 test groups (administered with different doses of the aqueous extract) and the A5 test group (fed with salinomycin mixed food) had significant differences with PC group in oocysts excretion and oocyst reduction rate ($P < 0.05$) (Figure 5).

Bloody diarrhea and cecal lesion value

All chicks infected with E. tenella oocysts developed bloody diarrhea, but the symptoms in the groups treated with medicine were lighter than those in the PC group. Especially, A5 group and A2, A3, and A4 groups were higher than that of the PC group ($P < 0.05$) (Table. 4 and Figure 6A). In addition, the cecal lesions status of the medicine feeding test group was significantly different from that of the PC group (Table 4 and Figure 6B).

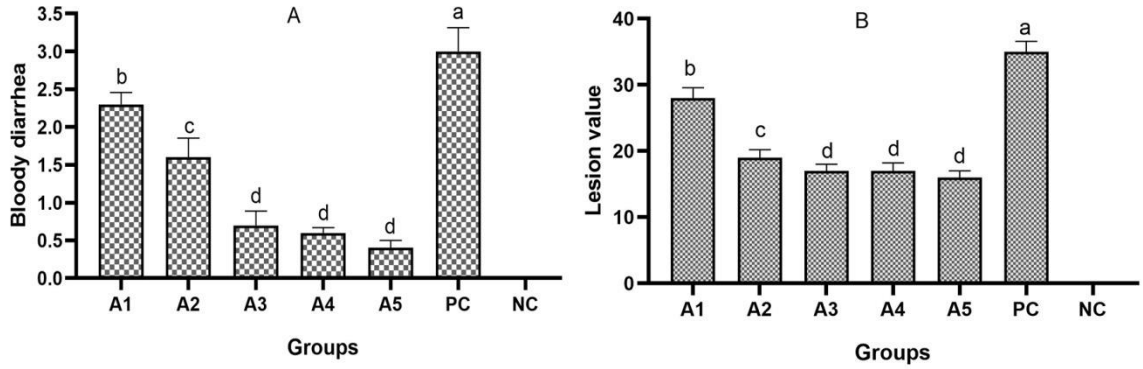

Figure 6. Effect of J. mandshuria extract on bloody diarrhea and cecal lesion value in chicks after oral infection with E. tenella oocysts. A1-A4: groups applied with aqueous extract of J. mandshurica epicarp; A1: 1 mL aqueous extract/kg bodyweight; A2: 3mL aqueous extract/kg bodyweight; A3: 5 mL aqueous extract/kg bodyweight; A4: 7 mL aqueous extract/kg bodyweight; A5: 66mg salinomycin/kg in feed; PC: positive control (infected without medication); NC: negative control (uninfected without medication). a–d Different letters indicate statistically significant differences ($P < 0.05$).

These results demonstrated that the aqueous extract of J. mandshurica epicarp has a positive effect on alleviating bloody Diarrhea and the pathological symptoms caused by coccidiosis.

Anticoccidial index

In comparison with the PC group, the test groups fed medicine showed a significant anticoccidial effect, especially the anticoccidial index of groups A3, A4, and A5 was 160 or higher, indicating that the anticoccidial effects were marked according to Yasuhiro *et al.* (1977), (Table. 4 and Figure 7).

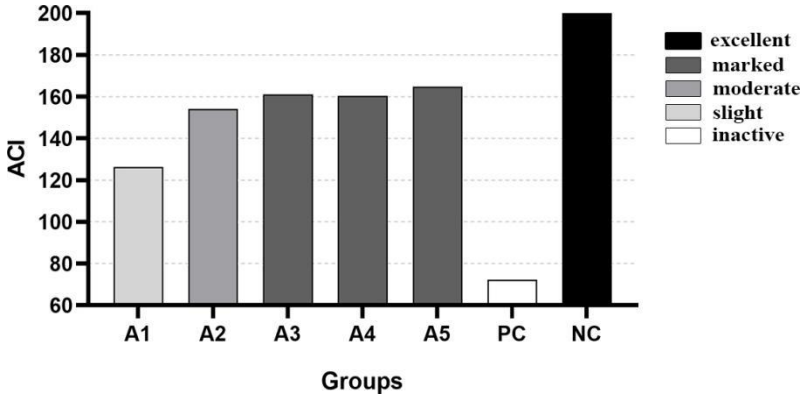

Figure 7. Effect of J. mandshurica extract on the anticoccidial index in chicks after oral infection with E. tenella oocysts. A1-A4: groups applied with aqueous extract of J. mandshurica epicarp; A1: 1mL aqueous extract/kg bodyweight; A2: 3mL aqueous extract/kg bodyweight; A3: 5 mL aqueous extract/kg bodyweight; A4: 7 mL aqueous extract/kg bodyweight; A5: 66mg salinomycin/kg in feed; PC: positive control (infected without medication); NC: negative control (uninfected without medication). These results illustrated that the aqueous extract of J. mandshurica epicarp has a good anticoccidial activity similar to salinomycin.

Discussion

With the increase in the use of veterinary drugs, the safety issue of livestock products has become a major concern. In particular, human health issues owing to the chemical drug residues in livestock products and reduced therapeutic effects due to drug resistance (Takele, 2016) are recognized as major challenges for the veterinary sector. As one of the ways to solve this problem, plant medicine development is very important in meeting people's demand for natural food and reducing the cost of medicine use (Nahed *et al.*, 2022; Tuhinadri and Samir, 2015). Many plant-derived drugs are currently being used in veterinary medicine, particularly as anticoccidial agents. Several plant-based anticoccidial agents have been introduced, such as Curcuma longa L (Abbas *et al.,* 2010), Rumex nervosus (Saleh *et al.,* 2020), Pinus radiata (Abdul *et al.,* 2009), and Brucea javanica (Lan *et al.,* 2016). These plant-based veterinary drugs are not only effective as anticoccidials due to their natural active ingredients, but also have a positive effect on the growth of broiler chickens (Abdul *et al.,* 2009; Abbas *et al.,* 2010; Saleh *et al.,* 2020). J. mandshurica has been widely used as a natural medicine to treat various diseases among people for a long time (An-dong *et al.*, 2019; Yuan-Yuan *et al.,* 2019). J. mandshurica plant organs are currently known to be enriched with more than 400 compounds, including quinones, phenolics, triterpenoids, diarylheptanoids, flavonoids, coumarins, lignans, phenylpropanoids, steroids, etc (Rami and Hyun, 2006; Yuwei *et al.,* 2016). In addition, various pharmacological activities of J. mandshurica were investigated in several studies, and it has been reported that it exhibits various biological activities such as anti-tumor, immunomodulatory, anti-inflammatory, neuroprotective, anti-diabetic, anti-viral, antibacterial and anti-melanin-producing activity (Yao *et al.,* 2012; Yongli *et al.,* 2013; Fei *et al.*, 2021).

Coccidiosis is a well-known protozoan disease in the poultry industry. This disease destroys the intestinal epithelial cells of chickens, which causes bloody diarrhea. When chickens are infected with coccidium, the resistance to the external environment decreases due to a decrease in the immune response, and they display typical clinical symptoms such as loss of appetite and depression (Chapman, 2014; Jabbar *et al.,* 2014). In addition, bloody diarrhea and oocyst leakage through feces are serious problems that can accompany secondary infection and the spread of coccidiosis in poultry (Anteneh *et al.,* 2019). According to the test results, it was found that the aqueous extract of J. mandshurica epicarp had anticoccidial properties by inhibiting oocyst activity and inhibiting sporulation. In some studies, the sporulation inhibition rates of leaf and fruit extract of P. emblica (1% concentration) were 35.7% and 36.7%, respectively (Usha *et al.,* 2021). This optimum concentration displays the highest inhibition rate (Usha *et al.,* 2021). However, the sporulation inhibition rates of the aqueous extract of J. mandshurica epicarp were 38, 54, 68 77, and 84% when the concentration were 1 mg/mL (1%), 10 mg/mL (10%), 30mg/mL (30%), 60mg/mL (60%), 100mg/mL (100%), respectively. This revealed that the inhibitory efficiency of the aqueous extract of J. mandshurica epicarp against coccidia increases linearly with the concentration increase.

In the broilers fed the aqueous extract of J. mandshurica epicarp, the symptoms of bloody diarrhea were significantly lower than that of the PC group, and there was a clear difference in the amount of oocysts excretion through feces. This might be due to the anti-inflammatory and antibacterial effects of the juglone component of J. mandshurica epicarp.

Relative body weight gain of experimental groups fed with the aqueous extract of J. mandshurica epicarp at 5mL/kg of body weight was higher than that of experimental groups fed the aqueous extract of J. mandshurica epicarp at 1, 3, 7mL/kg of body weight. This can be attributed to the various pharmaceutical ingredients of J. mandshurica (Yan *et al.,* 2019), which can promote the metabolic activity of the body. As the main medicinal ingredient here, Juglone is a phenolic compound for treating inflammation and infectious diseases. Juglone is active in hepatic metabolism and affects several metabolic pathways related to energy metabolism. Taking a small dose is beneficial to health. However, in some studies conducted on rat liver where Juglone was used in an excessive amount, it was found to interfere with liver energy metabolism by acting as an uncoupler of oxidative phosphorylation in isolated liver mitochondria (Saling *et al.,* 2011). In addition, overdosage or unreasonable use of Juglone can lead to some adverse reactions, such as nausea, vomiting, dizziness, dyspnea, palpitation, and even shock and death (Saling *et al.,* 2011; Huo *et al.,* 2017). This indicates that the aqueous extract of J. mandshurica epicarp can promote metabolic activity in vivo at an appropriate dose, but it might also cause some negative effects in vivo when it exceeds a certain amount. Further research in this area should be strengthened in the future. Nevertheless, the relative body weight gain of all experimental groups was significantly higher than the PC group and there were no significant differences in the degree of bloody diarrhea, amount of oocysts excretion through feces, and the relative body weight in the experimental group fed with the aqueous extract of J. mandshurica epicarp at 5mL, 7 mL/kg BW compared with the group fed salinomycin. In addition, the results of this study showed that the ACI of groups fed with the aqueous extract of J. mandshurica epicarp at 5mL, 7mL/kg of body weight was above 160, which displayed a marked anticoccidial effect.

We have proved that J. mandshurica epicarp has a good therapeutic effect on chick coccidiosis, and we

believe that it will become a new anticoccidial medicine because of its low cost, simple pharmaceutical processes, and high application value.

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