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RESEARCH ARTICLE
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Cytokine expression, immune responses, hematology and growth performance of Eimeria tennela infected broiler chickens treated with essential oils

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ABSTRACT

Abstract: Coccidiosis is a protozoan disease of poultry with high economic significance. In current study, a total of 50 caeca samples with a history of bloody diarrhea were collected. The samples were subjected to DNA extraction, followed by PCR of ITS-1 gene. It was observed that isolated Eimeria tenella was closely related to isolates of China, India, and the USA, with mild observable heterology in oligonucleotide sequences. The isolated Eimeria tenella was used in the experimental study. A total of 80 chicks were divided into the 4 groups from G1-G4. G1 was kept as negative control. The chicks in the G2 were treated with Eimeria tenella @ 4.0 ×105/ml. In the G3 the chicks were treated with Eimeria tenella and essential oil @ 0.25ml/L drinking water. In the G4 the chicks were treated with Eimeria tenella and Amprolium, @ 0.25ml/L. At different days samples were collected and different parameters were studied. A significant decrease in pathological lesions were observed in birds of infected and essential oil-treated groups. Similarly, the essential oil-treated group found a significant decrease in oocysts count; however, no significant difference was found in hematological values. The essential oil-treated group showed a decrease in mortality rate and improved weight gain and FCR. Eimeria tenella-infected birds had high values of interferon-gamma and IL-10 (interleukin 10) as compared to the normal group. In vitro, the essential oil also proved an anticoccidial agent as significantly sporulation of oocysts was reduced ($P<0.05$). We conclude that the essential oil blends

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can be used as an alternative measure to the chemotherapeutic agent to control the coccidiosis in the poultry industry.

Keywords: *Coccidiosis, Eimeria tenella, Essential oil, caeca, cytokines, hematology, performance*

INTRODUCTION

Coccidiosis, a deadly disease that affects chicken productivity and welfare is caused by a parasite of the genus Eimeria and Isospora [1]. Both belong to the phylum Apicomplexa, having a complex life cycle. Members of Apicomplexa mainly affect the intestines of mammals as well as birds [2]. For global nutrition, the Poultry industry is the fastest growing sub-sector of agriculture and thus a major driving force of the economy [3]. Among different poultry birds, chicken, is a major poultry bird which contributes greatly to agricultural production through the supplying eggs and meat [4]. However, chickens are prone to so many fatal diseases which hinder the productivity and compromise welfare resulting in high mortality in some case [1]. Coccidiosis is a protozoan disease with high economic significance. Increased mortality due to coccidiosis is still considered a threat to the poultry industry [5]. The anti-protozoan potential of terpenes present in essential oils has been documented [6].

Avian coccidiosis was controlled by anticoccidial drugs but the over use of these lead to the development of resistance in the Eimeria and raised public health safety concerns about the drug residues in meat [7]. So there is need of the natural alternatives to the coccidiosis control agents [8]. Essential oils classified as phytogenics are a mixture of aromatic substances from herbs and species. They have been used as an alternatives in feed antibiotics in monogastric animals due to their antimicrobial properties [9]. The inclusion of essential oils in broilers diet modified host immunity, stimulated growth and modified GUT health. Essential oils have been widely used in the poultry industry due to their antimicrobial and anticoccidial properties [10]. Essential oils also show anthelmintic and antiparasitic activities. Thus it was hypothesized that use of essential oils may be used as an effective alternative to control avian coccidiosis [11]. As for as we know this study is the first to test the anticoccidial activities of essential oils in coccidiosis chicken disease model.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the ethical committee of the Department of Biology, College of Sciences and Humanities, Prince Sattam Bin Abdulaziz University, Riyadh, Saudi Arabia, and the institutional review board. The department received prior approval from their ethical and biosafety committees. Animal experiments were approved by the Biology Department, University Committee. Broiler Chickens were maintained under special pathogen-free conditions and treated according to the guidelines of Prince Sattam Bin Abdulaziz University. All study is reported in accordance with ARRIVE guidelines.

Isolation and molecular detection of Eimeria tenella by PCR

A total of 50 caeca samples with a history of bloody diarrhea were collected, rinsed with normal saline to remove the fecal debris and, sample of rinsed caeca were homogenized and transferred to 35% sodium chloride (NaCl) solution to induce the floatation of oocysts [12]. The supernatant was separated to collect the oocysts and processed for DNA extraction, as described by [13].

DNA extraction from fecal samples

DNA was extracted as described by [13]. The following steps were performed to rupture the oocysts wall; (I) Freezing in liquid nitrogen: 50 times. (II)Thawing in a shaking water bath at 50°C: 50 times. 10ul solution was examined under the light microscope (40X) to confirm the complete oocysts wall rupture. After the complete lysis of the oocysts wall, DNA was extracted as described below, using the EZ-10

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Spin Column Animal Genomic DNA Miniprep Kit (Biobasic, Inc. Canada, Cat. No. BS427)

Quantification of Nucleic acid by Nano-drop

For the quantification of DNA, Nano-drop was used to check the concentration and purity of nucleic acid. Thermo Scientific Nano-Drop™ 2000/2000c Spectrophotometer was used for this purpose as described by [14].

Amplification of ITS1 gene

Amplification of isolated DNA was performed with a specific primer targeting the ITS1 gene to detect the Eimeria tenella (Table 1). The same primers were used for the negative control reaction mixture under the same amplification conditions as adopted by [15].

TABLE 1: Primer sequences used in a polymerase chain reaction

Primer	Oligonucleotide Sequence (5’-----3’)	Product Size
Forward	CCGCCCAAACCAGGTGTCACG	564 bp
Reverse	CCGCCCAAACATGCAAGATGGC	

Visualization of PCR amplicons

1.5% agarose gel with 5 µL of ethidium bromide was prepared and shifted to the electrophoresis tank already containing 1X TAE buffer. 5µL of each PCR reaction product and negative control samples were loaded in respective agarose gel wells with proper identity followed by loading of 5µL DNA markers in the first well. Through the agarose gel, 110 volts at 300 amperes for a minute whereas areas passed, after that gel was kept in the gel documentation system (Bio-Rad, USA) to visualize the PCR product band (positive sample). Gel lanes showing were considered positive samples while others with no bands were considered negative samples as described by [16].

A sequence analysis Gel extraction kit (Thermo Scientific GeneJET Gel Extraction Kit, Catalog No: K0691, Lot No: 0000240509) was used to purify the PCR product as described by Hamidinejat et al. (2010). The purified PCR product was further processed for gene sequencing.

Birds and housing

A total of eighty-day-old broiler chicks were purchased from a local hatchery. The chicks were housed in experimental sheds of the department of pathology, UVAS Lahore, for 28 days. The standard protocol was followed to rear the birds. Feed and water were available ad libitum throughout the experiment.

Experimental design

Birds were distributed into four groups; G1-G4. The dried floors of the four pens were covered with wood shavings from the same source. Group (G1) was considered a negative, with no infection and treatment. Group (G2) was positive control, infected at day 21 with sporulated oocysts of Eimeria tenella (4.0 ×105/ml) (Wang et al. 2018). Group (G3) was infected and treated with an essential oils blend (eucalyptus oil, peppermint oil, menthol, saponin, and liquid binder) at 0.25ml/L drinking water. Group (G4) was infected and treated with a standard drug (Amprolium, @ 0.25ml/L) (Shekhar et al. 2018) for comparative studies. All the birds were vaccinated for NDV on day1 and 14, and with bivalent IBV and IBDV on day8 [17]. The experimental design have been shown in the Table 2.

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TABLE 2: Experimental design

Groups	No. of birds	Treatment
G1	20	Negative control
G2	20	Positive control (Eimeria tenella @ 4.0 ×105/ml
G3	20	First treatment group (Eimeria tenella @ 4.0 ×105/ml) + (essential oils @ 0.25ml/L drinking water).
G4	20	Second treatment group (Eimeria tenella @ 4.0 ×105/ml + Amprolium, @ 0.25ml/L)

Oocyt counts

Oocyst count was performed according to the protocol as described by [18]. Briefly, caeca were rinsed with normal saline to remove the fecal debris. 1-2 grams of rinsed caeca were homogenized and transferred to a 35% sodium chloride (NaCl) solution to induce the floatation of oocysts. The supernatant was separated to collect the oocysts. Collected oocysts were floated into two compartments of the McMaster chamber, waiting for five minutes to settle down the fecal debris. Under a light microscope (10X magnification), the oocysts were counted within the grid of the two chambers, which were further multiplied by 50 to get the oocyst count per gram of the caeca [6].

Bloody in the feces was observed on different days and severity score was given, Severe (+++), mild (++) and moderate (+).

Hematological analysis

Hematological parameters were estimated as described by Benjamin [19].

Histopathological examination

Birds were sacrificed and macroscopic lesions on kidney and liver were noticed. These organs were fixed in 10 % neutral buffered formalin and processed for histopathological examination following the method of Bancroft and Gamble [20]. Morphological alterations were observed under light microscope.

Broiler performance responses

On days 1, 7, 14, 21, and 28, all chicks of each group were weighed to determine their body weight gain. The feed intake of each group was calculated by subtracting the residual feed from the offered feed. Following formula was used to calculate the feed conversion ratio;

Feed Conversion Ratio (FCR): Feed intake (g)/ body weight gain (g) [21]

Any bird that died was weighed, and the FCR values were calculated by dividing the total FI by BWG of live plus dead birds. Necropsies were performed on birds that died during the current study [22, 23].

Mortality rate

At the end of the trial mortality rate of each group was calculated and was converted into percentage form [24].

Determination of the level of chicken interferon-gamma (IFN-γ)

Interferon-gamma (IFN-γ) from the plasma samples was determined by using a chicken interferon-gamma (IFN-γ) quantitative sandwich ELISA Kit (catalog number: MBS700243).

Determination of the level of chicken interleukin 10 (IL10)

Interleukin 10 (IL10) level from the plasma samples was determined by using a chicken interleukin 10 (IL10) quantitative sandwich ELISA Kit (catalog number: MBS266842).

Statistical Analysis

The Mean± Standard deviation of all the parameters was compared among the different treatments by ANOVA followed by Tukey's test with the help of a statistical package of social sciences (IBM SPSS, version 23), reporting

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significant differences in means at P<0.05. The mortality frequencies were compared among the different treatments by the Chi-Square method.

RESULTS

This section may be divided by subheadings. It should provide a concise and precise.

Molecular detection of Eimeria tenella

The samples were subjected to DNA extraction, followed by PCR of ITS-1 gene. The oligonucleotide sequences were aligned and analyzed for phylogenetic analysis. It was observed that isolated Eimeria tenella was closely related to isolates of China, India, and the USA, with mild observable heterology in oligonucleotide sequences. The PCR result has been shown in the figure 1.

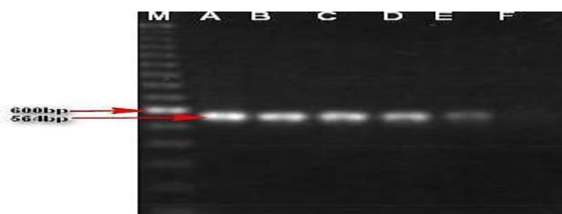

FIGURE 1: PCR results of Eimeria tenella Lane M: DNA Ladder 600 base pair, Lane A, B, C, D, E: Positive samples of Eimeria tenella (564 amplicon size), Lane F: Negative control

Oocyte Count

The oocyte count of different groups a various days has been shown in the figure 2.

Throughout the experiment, no oocyst was noted in group G1 as being negative control. On days 25, 26, 27, 28 and 29 oocysts count of group G2 (positive control) were 120726.10±12330.84, 188226.10±15414.76, 226692.30±57875.48, 152427.10±15414.75 and 70631.80±33755.12 respectively. On days 25, 26, 27, 28 and 29 oocysts count of group G3 (infected + treatment with essential oil blend) were 19148.30±2583.61, 37148.30±3110.60, 74037.80±3230.91, 29637.80±2110.6 and 1408.60±200.66 respectively which were significantly lower as compared to positive control. At day 25, 26, 27, 28 and 29 oocysts count of group G4 (infected + amprolium) were 11028.80±3146.63, 26778.80±2596.78, 59366.70±6852.74, 22161.30±2596.77 and 974.10±131.13 respectively which were significantly lower than control positive. The oocyte count of the G4 were also lower than G3 but that was non-significant control. Maximum no. of oocysts were counted in G2 followed by group G3 and least in group G4, although on days 25, 26, 28, and 29 groups G3 and G4 had no remarkable difference (P>0.05) in oocyst count.

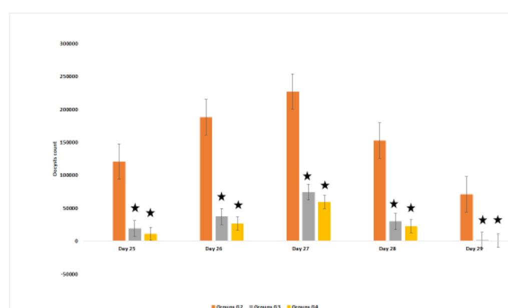

FIG 2: Oocyte count of the chicks at different days. Vertical error bars with stars are significantly different positive control.

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Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Dropping/fecal score

The dropping/ fecal score has been given in the Table 3.

Bloody diarrhea was observed in fecal samples of birds. Fecal samples of groups G3 and G4 had a mild quantity of blood, therefore, considered as (+). In contrast, fecal samples of birds of group G2 were tan colored with intense, bloody diarrhea hence considered as (++ to +++) dropping score. The maximum fecal score was observed in the group G2, followed by G3 and G3.

TABLE 3: Bloody diarrhea scores of different groups

Days	Groups			
	G1	G2	G3	G4
Day 25	-	+++	+	+
Day 26	-	+++	+	+
Day 27	-	+++	+	+
Day 28	-	++	+	+
Day 29	-	++	+	+

Severe (+++), mild (++) and moderate (+).

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Red blood cell count (1×106 cells per microliter)

The red blood cells count of the different groups at different has been given in the Table 4.

On day 21 red blood cell counts (1×106 cells/µL) of groups G1, G2, G3, and G4 were 2.99±0.25, 3.02±0.53, 2.86±0.23, and 3.06±0.28 respectively with no observable significance difference ($P>0.05$) between the groups. On day 24, red blood cell counts (1×106 cells/µL) of groups G1, G2, G3, and G4 were 2.96±0.31, 1.93±0.15, 3.03±0.41, and 3.08±0.31 respectively. G2 showed significantly lower values of red blood cells count as compared to G1 while all other groups were non-significant negative control. On day 26, red blood cell counts (1×106 cells/µL) of groups G1, G2, G3, and G4 were 3.09±0.23, 1.92±0.22, 3.05±0.33, and 2.84±0.30 respectively. While At day 26, red blood cell counts (1×106 cells/µL) of groups G1, G2, G3, and G4 were 2.98±0.26, 1.99±0.14, 3.00±0.31, and 3.08±0.31 respectively. Groups G2 expressed significantly ($P<0.05$) a low number of RBCs count (1×106 cells/µL) as compared to other groups. Similar trends were observed at day 28 as of 24 days.

TABLE 4: Red blood cells (1×106 cells per microliter) of different groups on different days

Days	Groups			
	G1	G2	G3	G4
Day 21	2.99±0.25	3.02±0.53	2.86±0.23	3.06±0.28
Day 24	2.96±0.31[a]	1.93±0.15[b]	3.03±0.41[a]	3.08±0.31[a]
Day 26	3.09±0.23[a]	1.92±0.22[b]	3.05±0.33[a]	2.84±0.30[a]
Day 28	2.98±0.26[a]	1.99±0.14[b]	3.00±0.31[a]	3.08±0.31[a]

Values with different letters are significantly different from control negative.

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Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Packed cell volume (%)

The packed cell volume values has been given in the Table 5.

On day 21, packed cell volume (%) of groups G1, G2, G3, and G4 were 43.50±9.08, 44.47±5.56, 37.10±8.69, and 43.88±6.43 respectively with no significant difference (P>0.05) between the groups. On day 24, packed cell volume (%) of groups G1, G2, G3, and G4 were 45.46±5.08, 24.42±1.57, 44.22±7.16, and 46.39±5.78 respectively. G2 showed significantly lower value as compared to all other groups. On day 26, packed cell volume (%) of groups G1, G2, G3, and G4 were 46.10±5.02, 25.11±1.74, 42.71±10.97, and 45.11±5.26 respectively. On day 28, packed cell volume (%) of groups G1, G2, G3, and G4 were 43.34±6.41, 24.33±1.18, 42.84±9.21, and 42.65±6.56 respectively. Group G2 had significantly (P<05) low packed cell volume (%) values as compared to other groups.

TABLE 5: Packed cell volume (%) of different groups on different days.

Days	Groups			
	G1	G2	G3	G4
Day 21	43.50±9.08	44.47±5.56	37.10±8.69	43.88±6.43
Day 24	45.46±5.08	24.42±1.57	44.22±7.16	46.39±5.78
Day 26	46.10±5.02 [a]	25.11±1.74 [b]	42.71±10.97 [a]	45.11±5.26 [a]
Day 28	43.34±6.41 [a]	24.33±1.18 [b]	42.84±9.21 [a]	42.65±6.56 [a]

Values with different letters are significantly different from control negative.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Hemoglobin concentration

The values of Hb concentration of the different groups at different days has been given in the table 6.

On day 21, hemoglobin (Hb) concentration (g/dL) of groups G1, G2, G3, and G4 were 8.97±0.67, 8.25±0.45, 8.58±0.78, and 8.18±0.73 respectively. All the groups were non-significant to control negative. On day 24, hemoglobin (Hb) concentration (g/dL) of groups G1, G2, G3, and G4 were 8.03±0.90, 5.75±0.72, 8.18±0.42, and 8.76±0.89 respectively. G2 showed significantly lower Hb concentration as compared to all other groups. On day 26, hemoglobin (Hb) concentration (g/dL) of groups G1, G2, G3, and G4 were 8.97±0.65, 6.64±0.68, 8.93±0.93, and 8.93±0.69 respectively. On day 28, hemoglobin (Hb) concentration (g/dL) of groups G1, G2, G3, and G4 were 8.15±0.64, 5.25±0.85, 8.75±0.75, and 8.59±0.15 respectively. At day 26 and 28 G2 showed significantly lower Hb concentration as compared all other groups.

TABLE 6: Hemoglobin (Hb) concentration (g/dL) of different groups on different days

Days	Groups			
	G1	G2	G3	G4
Day 21	8.97±0.67	8.25±0.45	8.58±0.78	8.18±0.73
Day 24	8.03±0.90 [a]	5.75±0.72 [b]	8.18±0.42 [a]	8.76±0.89 [a]
Day 26	8.97±0.65 [a]	6.64±0.68 [b]	8.93±0.93 [a]	8.93±0.69 [a]
Day 28	8.15±0.64 a	5.25±0.85 [b]	8.75±0.75 [a]	8.59±0.15 [a]

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Values with different letters are significantly different from control negative.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Leukocytes count (×103/µL)

The leukocyte count of different groups at different days has been shown in the Table 7.

On day 21, leukocytes count (×103/µL) of groups G1, G2, G3, and G4 were 12.52±0.78, 15.54±5.8, 14.76±4.28, and 14.38±7.98 respectively. On day 24, leukocytes count (×103/µL) of groups G1, G2, G3, and G4 were 13.25±0.39, 17.98±4.9, 14.67±5.64, and 14.75±4.29 respectively. On day 26, leukocytes count (×103/µL) of groups G1, G2, G3, and G4 were 12.78±0.25, 19.75±4.5, 14.68±7.25, and 13.98±6.21 respectively. On day 28, leukocytes count (×103/µL) of groups G1, G2, G3, and G4 were 13.54±0.41, 21.91±6.9, 14.28±6.98, and 15.21±5.32 respectively. There was no significant difference (P>0.05) observed regarding the leukocyte count. At 21 day leukocyte count of all the was not significant to control negative group while at day 24, 26 and 28 G2 showed significantly higher values of leukocytes as compared to the all other groups.

TABLE 7: Leukocytes count (×103/µL) of different groups on different days

Days	Groups			
	G1	G2	G3	G4
Day 21	12.52±0.78	15.54±5.8	14.76±4.28	14.38±7.98
Day 24	13.25±0.39 [a]	17.98±4.9 [b]	14.67±5.64 [a]	14.75±4.29 [a]
Day 26	12.78±0.25 [a]	19.75±4.5 [b]	14.68±7.25 [a]	13.98±6.21 [a]
Day 28	13.54±0.41 [a]	21.91±6.9 [b]	14.28±6.98 [a]	15.21±5.32 [a]

Values with different letters are significantly different from control negative.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Intestinal Lesion Score

Intestinal lesion score of different groups has been shown in the figure 3.

Group G1 had no intestinal lesions on gross examination. On days 24, 26, and 28, the intestinal lesion score of group G2 (positive control) was 2.20±0.84, 3.20±0.84, and 2.33±0.57, respectively. On days 24, 26, and 28, intestinal lesion scores of group G3 (infected + treatment with essential oil blend) were 1.20±0.45, 1.60±0.55, and 1.00±0.00 respectively. On days 24, 26, and 28, intestinal lesion scores of group G4 (infected + amprolium) were 0.80±0.00 1.33±0.45, and 0.93±0.57 respectively. The highest gross pathological lesions were observed in group G2, followed by group G3 and least in group G4. A significant decrease (P<0.05) in intestinal lesions score was observed in infected treated and non-treated birds. G3 and G4 showed significantly lower intestinal lesion score as compared to positive control at day 24, 26 and 26.

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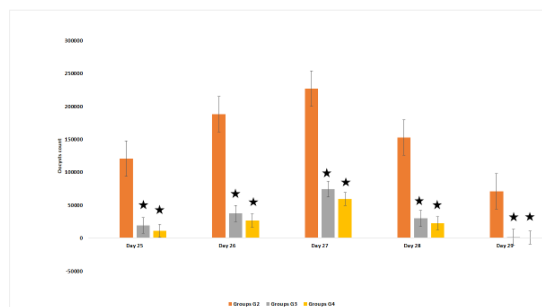

FIG 3: Intestinal lesion scoring at different days. Vertical error bars with stars are significantly different from positive control.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Histopathological Examination

Histopathological lesion scoring of the different groups at different days has been shown in the figure 4.

It was observed that Group G1 had no microscopic lesions when examined under a light microscope. On days 24, 26, and 28, the microscopic lesions score of group G2 (positive control) was 3.33±0.82, 3.10±0.74 and 2.40±0.52 respectively. On days 24, 26, and 28, microscopic lesions score scores G3 (infected + treatment with essential oil blend) were 1.07±0.59, 0.90±0.57, and 0.70±0.48, respectively. On day 24, 26 and 28 microscopic lesions, scores of group G4 (infected + amprolium) were 0.67±0.49, 0.50±0.53 and 0.30±0.48 respectively. The highest microscopic lesions score was observed in group G2, followed by group G3 and least in group G4. A significant difference ($P<0.05$) in microscopic lesions score was observed in infected treated and non-treated birds.

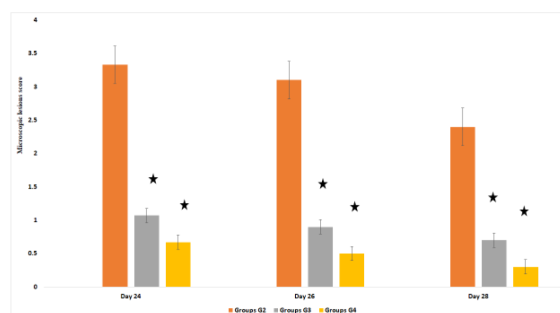

FIG 4: Microscopic lesion scoring of the chicks at different days. Vertical error bars with stars are significantly different from positive control.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Microscopic changes

Microscopic changes in the caeca of birds of different groups has been shown in the Fig 5.

The caeca of the G1 (negative control) was normal and did not show any microscopic change. The caeca of the G2 (positive control) showed severe sloughing of epithelium and degenerative changes while mild to moderate microscopic changes were observed in the caeca of group G3 and G4.

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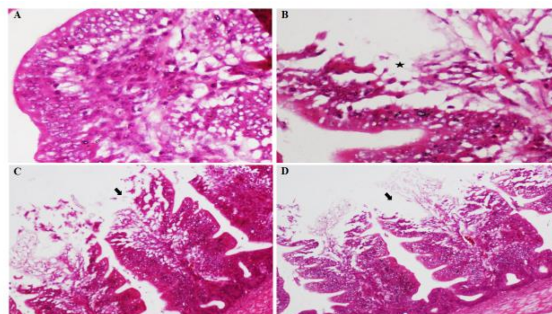

FIG 5: Microscopic changes in the caeca of the chicks infected with Eimeria and treated with essential oil and amprolium. A; normal caeca of control negative (G1). B; caeca of positive control (G2) showing severe necrotic and degenerative changes. C; Caeca of combination group infected + Essential oil treated (G3), showing mild degenerative changes; D; Caeca of the second combination group infected + amprolium (G4); showing mild degenerative changes.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Body weight gain

Body weight gain of the different at different days has been given in the Table 8.

No significant difference (P>0.05) was observed between the groups in body weight on days 1, 7, 14, and 21. While at day 28, the body weight of groups G1, G2, G3, and G4 were 1214.80±27.20, 924.40±39.81, 1159.10±14.12, and 1186.10±17.89 respectively. The highest body weight gain was observed in negative control group G1 followed by group G4 and least in positive control group G2.

TABLE 8: Comparison of broiler body weight gain of different groups on different days

Days	Groups			
	G1	G2	G3	G4
Day 1	42.60±2.01	42.90±1.72	42.10±2.02	42.20±2.39
Day 7	133.60±8.19	122.50±5.96	120.60±8.42	130.80±8.62
Day 14	363.70±13.69	353.60±11.88	332.40±10.95	346.90±11.68
Day 21	735.70±15.71	628.50±18.16	725.30±7.87	730.20±12.07
Day 28	1214.80±27.20[a]	924.40±39.81b	1159.10±14.12[a]	1186.10±17.89[a]

Values with different letters are significantly different from control negative.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Feed conversion ratio

Feed conversion ratio of the different groups at different days has been given in the Table 9.

On day seven the feed conversion ratio of groups G1, G2, G3, and G4 were 0.952±0.017, 1.002±0.020, 0.963±0.016, and 0.958±0.012 respectively. On day 14 feed conversations of groups G1, G2, G3, and G4 were 1.137±0.035, 1.204±0.012, 1.142±0.016, and 1.141±0.017 respectively. On day 21 the feed conversion ratio of groups G1, G2, G3, and G4 were 1.367±0.041, 1.525±0.029, 1.419±0.039, and 1.391±0.034 respectively. No significant difference (P>0.05) was observed between the groups in the feed conversion ratio on days 7, 14, and 21. While at

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day 28 the feed conversion ratio of groups G1, G2, G3, and G4 were 1.459±0.030, 1.716±0.038, 1.574±0.038, and 1.504±0.042 respectively.

TABLE 9: Comparison of feed conversion ratio of different groups on different days

Days	Groups			
	G1	G2	G3	G4
Day 7	0.952±0.017	1.002±0.020	0.963±0.016	0.958±0.012
Day 14	1.137±0.035	1.204±0.012	1.142±0.016	1.141±0.017
Day 21	1.367±0.041	1.525±0.029	1.419±0.039	1.391±0.034
Day 28	1.459±0.030[a]	1.716±0.038[b]	1.574±0.038[a]	1.504±0.042[a]

Values with different letters are significantly different from control negative.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/L drinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Mortality Rate

Mortality of the groups has been shown in the figure 6.

At the end of the trial, the number of dead birds in groups G1, G2, G3, and G4 were 2, 19, 4, and 2, respectively. Hence the percentage mortality rate in groups G1, G2, G3, and G4 was 4.44, 42.22, 8.89, and 4.44, respectively. Regarding the mortality rate, a non-significant difference (P<0.05) was observed between the groups.

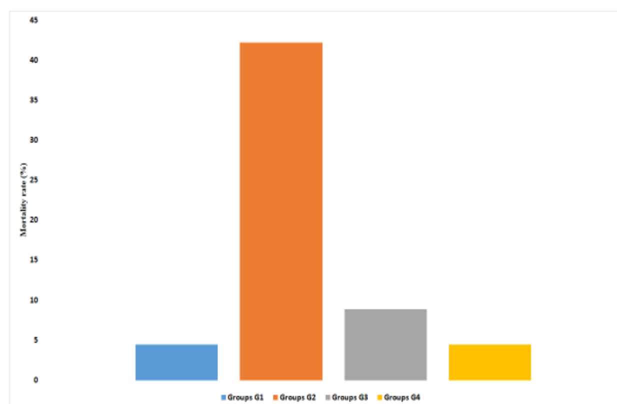

FIG 6: Mortality percentage of the different groups.

Treatments: G= negative control, G2=Eimeria tenella @ 4.0 ×105/ml, G3= Eimeria tenella and essential oil @ 0.25ml/Ldrinking water. G4=Eimeria tenella and Amprolium, @ 0.25ml/L.

Interferon-gamma (IFN-γ) level in birds

Interferon-gamma (IFN-γ) concentrations of the different groups have been shown in the figure 8.

On day 21 interferon-gamma (IFN-γ) levels in birds of groups G1, G2, G3, and G4 were 9.100±0.85, 9.050±0.83, 8.950±0.83, and 8.700±0.80 pg/mL respectively with no significant observable difference (P>0.05) between the groups. On day 24 interferon-gamma (IFN-γ) levels in birds of groups G1, G2, G3, and G4 were 9.850±0.81, 51.050±5.25, 28.950±3.12, and 29.250±2.36 pg/mL respectively. On day 26 interferon-gamma (IFN-γ) levels in birds of groups G1, G2, G3, and G4 were 9.300±0.80, 53.35±8.83, 20.25±2.64, and 19.40±3.31 pg/mL

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respectively. While At day 28 interferon-gamma (IFN-γ) levels in birds of groups G1, G2, G3, and G4 were 8.800±0.89, 39.50±5.63, 18.50±3.68, and 20.24±3.29 pg/mL respectively. Significantly high (P<0.05) interferon-gamma (IFN-γ) level was noted in birds of group G2 followed by birds of groups G3, G4, and normal values in group G1.

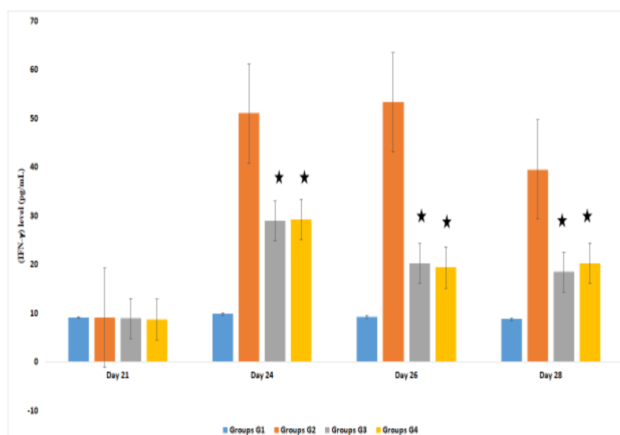

FIG 7: Interferon-gamma (IFN-γ) concentrations of the different groups. Vertical error bars with stars are significantly different from positive control.

Interleukin-10 (IL-10) level in plasma sample of birds

Interleukin-10 (IL-10) concentration of different groups have been presented in the figure 9.

On day 21 interleukin-10 (IL-10) levels in birds of groups G1, G2, G3, and G4 were 8.12±0.65, 8.02±0.73, 8.91±0.93, and 8.42±0.69 pg/mL respectively with no significant observable difference (P>0.05) between the groups. On day 24 interleukin-10 (IL-10) levels in birds of groups G1, G2, G3, and G4 were 8.57±0.78, 47.54±5.78, 23.23±4.52, and 25.36±2.78 pg/mL respectively. On day 26 interleukin-10 (IL-10) levels in birds of groups G1, G2, G3, and G4 were 9.8±0.69, 50.32±7.43, 23.51±3.64, and 23.54±4.25 pg/mL respectively. While At day 28 interleukin-10 (IL-10) in birds of groups G1, G2, G3, and G4 were 9.6±0.85, 40.25±6.89, 19.34±3.47, and 21.98±2.86 pg/mL respectively. Significantly high (P<0.05) interferon-gamma interleukin-10 (IL-10) level was noted in birds of group G2 followed by birds of groups G3, G4, and normal values in group G1.

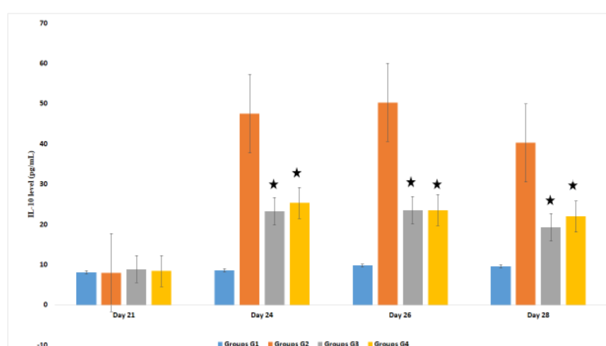

FIG 8: Interleukin-10 (IL-10) concentration of different groups. Vertical error bars with stars are significantly different from positive control.

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DISCUSSION

It is a well-established fact that, our planet earth is experiencing a continuous growth in human population. The current human population is around 8 billion and has been projected to a population to around 8.5 billion in 2030, 9.7 billion in 2050 and 10.4 billion in 2100. For this over-growing population, shelter, food and clothing become imperative. Food production carries a larger percentage of these basic human needs as its availability determines the existence of human population [25]. In view of this, much demand has been on the agricultural sector of each nation to increase food production that is safe for human consumption that will meet the ever-growing population.

Chicken, being the most important bird in poultry sector is supposed to be prone to more diseases and leading to life shortening phenomenon which ultimately affect its productivity to the consumers. Chicken coccidiosis is an enteric disease that impairs growth and suppresses the immune system resulting in high mortality which has been estimated to cost more than US$3 billion annually in poultry industry [1]. The disease is caused by protozoan apicomplexan parasites of genus Eimeria which consist of over 1000 species [1]. The essential oil has been reported for its anti-parasitic activity in the literature [26-28]. Together with this, the coccidiostat activity of terpenes present in other essential oils against Eimeria tenella in poultry has a paucity of documentations [29-30]. Here we tried to use essential oil as an alternative therapeutic agent for coccidiosis treatment in broiler chickens. We have found a significant decrease in oocytes counts in our experimental treated group compared to the infected group. This growth promotion is most likely due to the inhibitory effect of active ingredients of the terpenes against the microorganisms causing coccidiosis which are in accordance with previously published results [6]. Similarly, the bloody diarrhea and RBC count were significantly lower in our treatment group compared to the infected group which is again suggesting the inhibitory effect of essential oil against coccidiosis. These findings clearly demonstrate that treatment of essential oil has a clear inhibitory effect on Eimeria tenella which can be seen from the low packed cell volume, comparative hemoglobin (Hb) concentration, comparative leukocyte count, decrease in intestinal lesions score, and significant difference in microscopic lesions score in treated group compared to positive control group. Previous works documented the inhibitory effects of terpenes present in essential oils of eucalyptus and peppermint against avian bacteria [6, 27, 31, 32] and avian viruses [6, 33]. It is also worth noting that the growth-promoting effects of essential oils in broilers have been previously reported by [34]. Furthermore, we also observed a significant weight gain in our treated group compared to the non-treated group. This improvement might be due to the effect of essential oil on inhibition of multiplication of environmental micro-organisms as well inhibition of Eimeria tenella in treated group. We have also found a significant increase feed conversion ratio in treated compared to the non-treated group. However, we have found a non-significant mortality rate among the groups. It has been documented previously that, the benefits of control of Eimeria tenella infection in broilers on feed conversion ratios [35]. The standard addition of coccidiostats in the feed of broilers for 80% of the life of the bird with the withdrawal of the coccidiostats before slaughter is a globally adopted protocol in the broiler industry. We have also did experiments to check the the immune response of broilers experimentally infected with Eimeria tenella. Previously, the use of probiotics in feed is used to improve the immune system of the chicken against infection. Min reported the effectiveness of Lactobacillus-based probiotics, Primalac which reduced the oocyst shedding and increase T and B cellspecific cytokine against E. avervulina infection [35]. Here we found a significantly high IFN-γ and IL-10 level in treatment group which is suggesting an efficient immune response due to essential oil treatment. Our results were in accordance with the previously published data and suggests that the use of essential oil can improve the productivity of broiler chickens and protect them from infection of coccidiosis. This work will help to open new ways in understanding the use of

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different natural and essential oil and it therapeutic affect to replace the chemotherapeutic agent and use it as an alternative therapy.

CONCLUSIONS

From this study, it can be concluded that plant based resources have a beneficiary impact on the regulation and control of disease with no documented adverse effect on growth performance, hematological parameters, carcass quality, and immune response of chicken. It can be suggested that the combination of essential oil blend and standard disinfectant has the potential to protect broiler chickens against coccidiosis-induced alteration in growth and immune response parameters.

Author Contributions

Conceptualization, Qwait AlGbbani and Rajaa Abdulhadi Alhaddad; Data curation, Badreyah Diweihi alanezi and Lamia Yousef Ali AlGhilan ; Formal analysis, Qwait AlGbbani & Hailah M. Almohaimeed ; Investigation, Samia S. Alkhalil & Mona H.Soliman. Methodology, Mariam S. Al-Ghamdi & Amal abdulrahman almehini , Lamia Yousef Ali AlGhilan and Nawal Awadh Alonazi; Project administration, Rajaa Abdulhadi Alhaddad; Resources, Rajaa Abdulhadi Alhaddad & Hailah M. Almohaimeed; Software, Hailah M. Almohaimeed & Mona H.Soliman.Validation, Qwait AlGbbani & Mona H.Soliman; Visualization, Amal abdulrahman almehini; Writing – original draft, Qwait Al Gabbani & Mona H.Soliman; Writing – review & editing, Qwait Al Gabbani.All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

Authors declare no conflict of interest.

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