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Immunotherapeutic Efficacy of Dietary Black Cumin Seed Synergistic Action of Curcumin Experimental *Eimeria Tenella* Infections in Chicks

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Abstract

Coccidiosis is a major health and economic problem in the poultry industry. This work was planned to study the effect of *Nigella sativa* grinded seeds and Curcumin on experimental infection of chicks with *Eimeria tenella*. Chicks were divided into six groups; G (1) received *N. sativa* 500 part million (ppm), and curcumin 500 ppm, G (2) received *N. sativa* 1000 ppm, G (3) received Curcumin 1000 ppm, G (4) received anticoccidial drug (diclazuril), G (5) received no herbs or drugs as a control infected and G (6)10 normal non-infected control. All chicks were infected at 28 days old with 20,000 sporulated *Eimeria tenella* oocysts. Daily counting of oocysts was done for eight days post infection (p. i.). Chicks were weighed during the six weeks of the experiment. Blood was collected at 30 and 42 days for total WBCs count and total protein determination. Caeca from chicks were collected and preserved in 10% formalin to see gross pathological changes and (6µm) sections for histopathological examination. The results of this work showed that group (1) had a higher body weight gain from 14 to 21 d of age. Also, it had total WBCs count as normal healthy birds (32,250± 3,218 /µl) and a total serum protein (5.5 ± 1.2 g/dl). However, after experimental infection with *Eimeria tenella*, group (2) had higher body weight (1767.2 ± 64.1g). Also, total WBC count as normal healthy birds (57,750 ± 5,687/ µl), a total serum protein (5.1 ± 1.15 g/dl) and the lowest oocyst count. Histopathological examination during the recovery phase in *Nigella* ration showed mild infection of schizonts and inflammation of all layers of intestinal wall with crypt abscess composed of *Eimeria* schizonts and chronic inflammatory cells and parasitic granuloma composed of chronic inflammatory cells and fibrosis. These results are statistically significant. Thus, it can be concluded that *Nigella sativa* and curcumin have significant anticoccidial activity comparable to/ or better than Diclazoril.

Keywords: Coccidia; Infection; Pathogenicity; Immunotherapeutic

Introduction

Coccidia are a group of intracellular opportunistic protozoa which contain different genera. They share general features in morphology, life cycle and pathogenicity. Isospora is a human intestinal coccidian closely related to members of the genus Eimeria. Eimeria causes disease in various domestic animals, fish and birds [1]. Eimeria

causes the most widespread health problem in the broiler industry and remains one of the most expensive diseases of commercial poultry production [2-4]. The introduction of natural herbs into feeding programs also proved to have beneficial effect on poultry health and production [5]. For preventive therapy, coccidiostatics should be used for long time which may interfere with development of immunity and drug resistance also they may depress egg production and increase embryonic mortality. These drugs may have a bad effect on the consumers due to residual effects in poultry products '' meats and eggs''. Hence, they should be withdrawn at least 4 days before slaughtering ''broilers'' and 4 weeks before commencement of egg production [6]. *N. sativa* have been used in folk medicine in the Middle and Far East as a traditional medicine for a wide range of illness. The original research articles have shown the potential immunomodulatory and immunotherapeutic potentials of *N. sativa* seed active ingredients, in particular thymoquinone (TQ). The immunotherapeutic efficacy of TQ is linked to its antitoxic, anti-histaminic and anti-inflammatory properties. These effects with its immunomodulatory properties can explain the anti-microbial and anticancer properties of *N. sativa* oil or TQ. Schistosomiasis, a tropical parasitic disease, is endemic in the third world countries. Protection from this disease is mediated by both cellular and humoral immunity [7]. N. sativa seeds in a ratio of 2% in feed of poultry showed that it has an immuno-stimulant and growth promoter effects and Anti-helmenthic effect. AL- sayed, [8] found that it stimulates immunity by initiationand increase in cellular immunity and humoral immunity. Also, it has improved the immune response in vaccinated chickens against coccidial infection [9]. *Nigella sativa* was reported to have anticoccidial, vaccine promoting, growth promoting [10] and antihelminthics [7] effects. Curcumin was used as a nematocidal [11] and anti-inflammatory [12] and as antioxidant [13]. Furthermore, the plant was effective in reducing upper- and mid-small intestinal infections caused by *E. acervulina* and *E. maxima* [14]. Because of the difficulty in cycling Isospora than Eimeria, the present study was conducted to determine the effect of addition of some natural herbs (*Nigella sativa* Grinded Seeds and Curcumin powder) in chicks infected with Eimeria tenella isolates as an example of the coccidia family.

Materials and Methods

Experimental animals: 160clinically healthy broiler chicks, Cobb 308 hybrids, one day old and weighing 40 gm; the birds were housed in cages on slat floors under conditions excluding further Eimeria infection. The birds received a standard diet without antibiotics or coccidiostatics given ad libitum access to water and un-medicated starter feed.

Chicks were divided into 6 groups:

- Group (1): 30 chicks infected and received *Nigella sativa* at a dose of 500 ppm and Curcumin at a dose of 500 ppm.
- Group (2): 30 chicks infected and received *Nigella sativa* at a dose of 1000 ppm. Group (3): 30 chicks infected and received Curcumin at a dose of 1000 ppm.
- Group (4): 30 chicks infected and treated with anti-coccidial drug ''Diclazuril'' as a reference control treated group.
- Group (5): 30 chicks infected and received no treatment and serve as infected control.
- Group (6): 10 chicks non-infected and serve as a normal control.
- The experimental feed was a starter feed with non-anticoccidial additives. The feed was mixed with *Nigella sativa* powder and Curcumin as experimental protocol.

Parasites and Experimental Infection:

Eimeria tenella strain was isolated from infected birds and propagated at the animal housing facility of the poultry project in Libya. Sporulated oocysts were isolated in 2.5% sodium hypochlorite, washed three times with dist. water, and counted using a McMaster slide under a light microscope. All chicks in groups (1 – 5) were inoculated with 20,000 sporulated *E. tenella* oocysts in 0.5 ml water/ each at 28th day old. The 4th and 5th groups were infected and received complete ration free from additive as reference treated and control non-treated groups, respectively.

- All chicks were subjected to the following:

1. Parasitological Studies: Oocysts index measured, Daily oocysts count in excreta 5 times / day for eight days after the infection. In order to determine parasitological parameters, lesion scores per bird were assessed according to Johnson and Reid [15] and the oocyst index measured [16]. Counting coccidial oocysts (Eimeria spp.) has been performed according to the procedure given by Taylor, et al., [17] employing the commonly used variation of using gauze rather than a sieve to screen the faeces [18]. Each test sample was prepared and counted by using McMaster counting slide. Five samples were taken from each cage along the 24 h at 6, 10, 14, 18 and 22 h and the mean number is calculated / day.

2. Blood was Collected at 30 and 42 days for Total Leucocytic Count [19].

3. Examination of Pathological Changes in Caeca: Caeca from chicks were collected from the age of 32 to 45 days and preserved in 10% formalin to see gross pathological changes and 6 µm sections from infected and healthy chicks were prepared for histopathological examination. Caecal samples from each chick (~25mm in length) were fixed in 10% neutral buffered formalin for 48 hours and washed then placed in 70% ethyl alcohol (EtOH) for preparation of

paraffin sections to see the changes in the caeca. Staining the sections with H & E. stain and microscopic examination to see the histopathological changes were done according to Ahlqvist and Anderson [20].

Statistical Analysis: Statistical analysis of the results was done by using SPSS [21] Version 8.0 software. After one-way analysis of variance, Tukey's multiple range test (HSD) was used to test significance between different treatments at $P \leq 0.05$.

Results

Mortality and necropsy records: The total number of deaths in the experimental groups was 6 in Group (1), 5 in Group (2), 6 in Group (3), 9 in Group (4) and 9 in Group (5) with a total of 35 (Tables 1,2 and Figures 1-8).

Oocyst Count

Days Post Infection (X 10^5)/ Day								
Group	1st	2st	3st	4st	5st	6st	7st	8st
1	3 ± 0.28	2.5c ± 0.38	0.5c ± 0.08	0.225b ±0.09	.250 ±0.06	4.00Dc ± 0.45	5.00b ± 0.22	2.00b ±.32
2	1.25d ± 0.14	1.5c ± 0.14	0.5c ± 0.08	0.25b ± 0.07	0.25 ± 0.05	1d ± 0.11	2.5d ± 0.24	3.5b ± 0.22
3	2.5b ± 0.16	3c ± 0.16	0.25c ± 0.07	0.25b ± 0.07	0.3 ± 0.08	3.5c ± 0.47	6bc ± 0.27	4b ± 0.32
4	2.7a ± 0.25	10b ± 0.84	3. b ± 0.16	0.25b ± 0.05	0.25 ± 0.05	17.5b ± 0.55	7.5a ± 0.32	2.5b ± 0.32
5	3.75c ± 0.07	20a ± 0.70	3.75a ± 0.29	1a ± 0.08	0.75 ± 0.11	30a ±1.38	12.5d ± 0.55	7.7a ± 0.37
prop	***	**	**	**	NS	***	***	**

Table 1: Days Post Infections among different groups.
a,b,c,d Means with different superscripts are statistically different within the same column. (**P ≤0.01 ***P ≤0.001 NS: Not Significant).

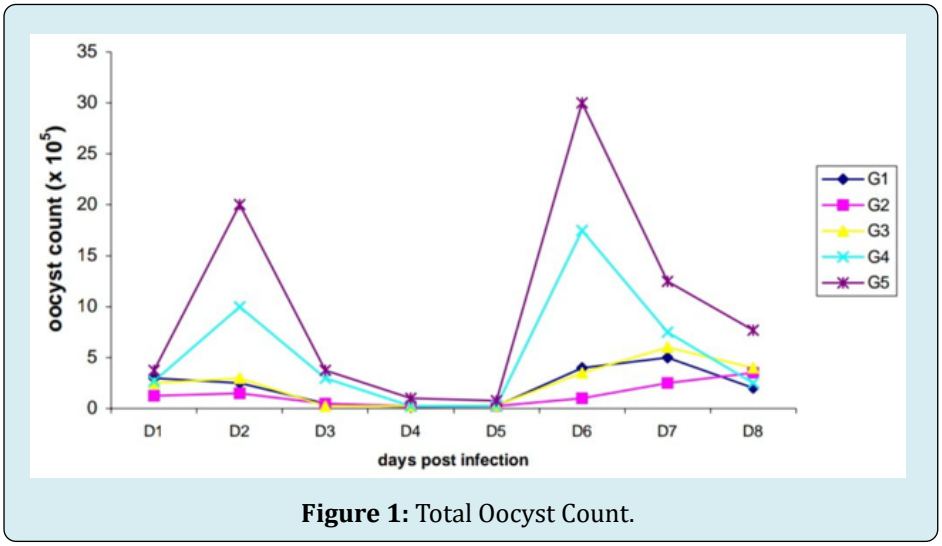

Figure 1: Total Oocyst Count.

Total White Blood Cell Count

WBCS Count	Experimental Groups					
	G1	**G2**	**G3**	**G4**	**G5**	**Prob**
30th day	3500^{c}±270.7	20.500^{b}±860.5	$13,550^{c}$±677.8	$10,800^{a}$±590.6	$28,750^{a}$±1170	***
42nd day	$32,250^{c}$±3,218	57.750^{c}±5,687	$69,600^{a}$±7,128	$54,900^{a}$±5,461	$90,250^{a}$±8,513	***

a,b,c,d,e Means with different superscripts are statistically different within the same row. ***P≤0.00
Table 2: Total WBCs count among different groups.

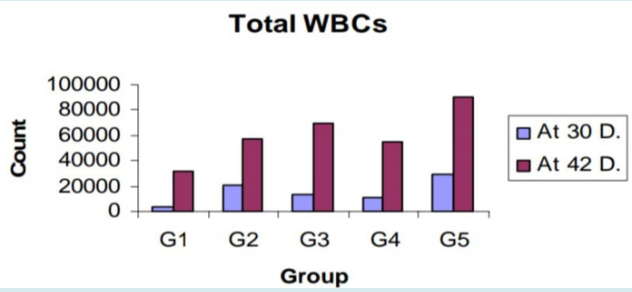

Figure 2: Total WBCs count among different groups at 30 and 42 days of age.

Postmortem Examination of Chicks' Coeca (Macroscopic Lesion)

Macroscopic lesions are presented in Figures 3-6. There were haemorrhages and necrosis affecting the two caeca of infected chicks.

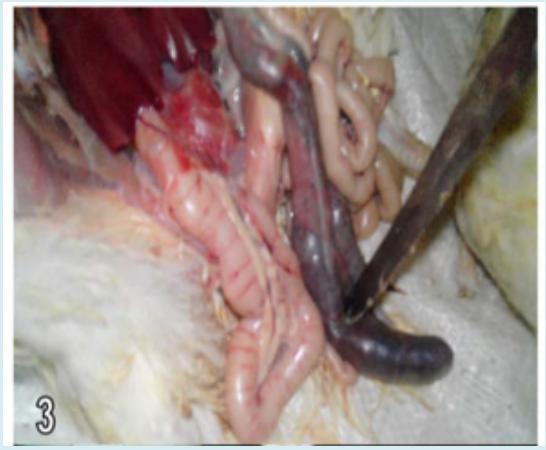

Figure 3: Caeca distended with blood from control chicks of group (5) at the age of 42nd day.

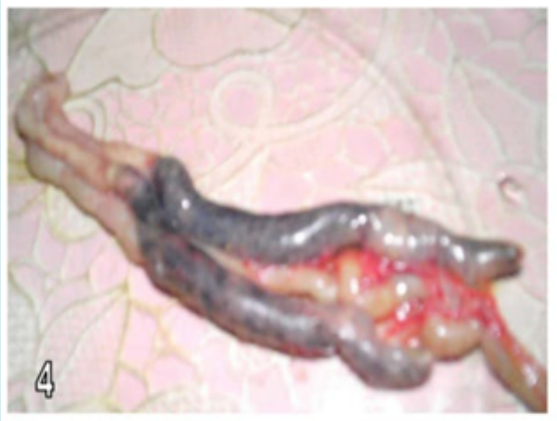

Figure 4: Caeca distended with blood in chicks of group (4) at the 6th day post infection.

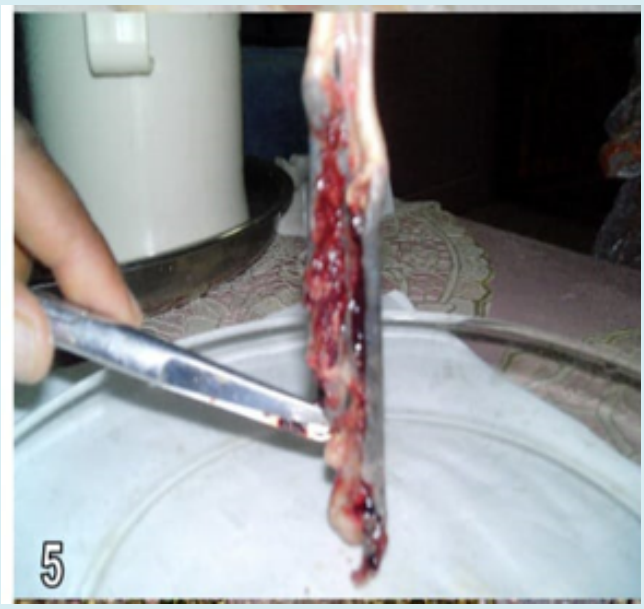

Figure 5: Caeca opened to show haemorrhagic debris. There may be firm caecal cores in subacute cases or that recovering in control chicks.

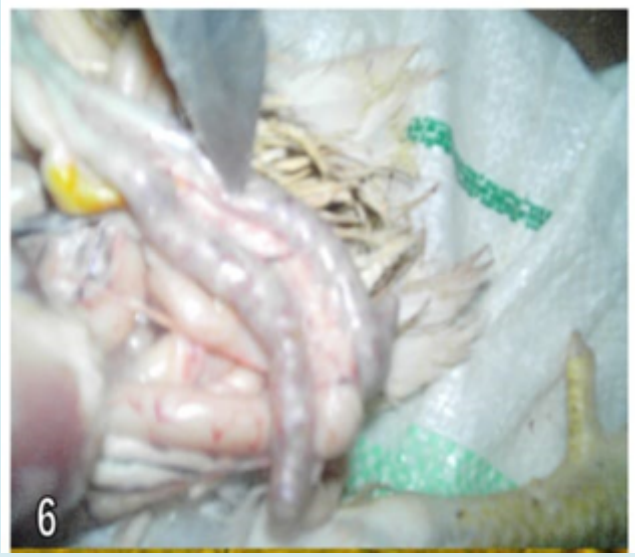

Figure 6: Caeca of experimental chicks (group 2) after 8th day of infection showing healing and slight Necrosis.

Microscopic Examination of Excreta and Mucosal Scraping:

Examination of excreta and mucosal scraping in the different groups reveals that the oocyst has the size of *E. tenella* oocyst (19 x 22 µm). The test groups vary in the intensity of infection (Figures 7 & 8).

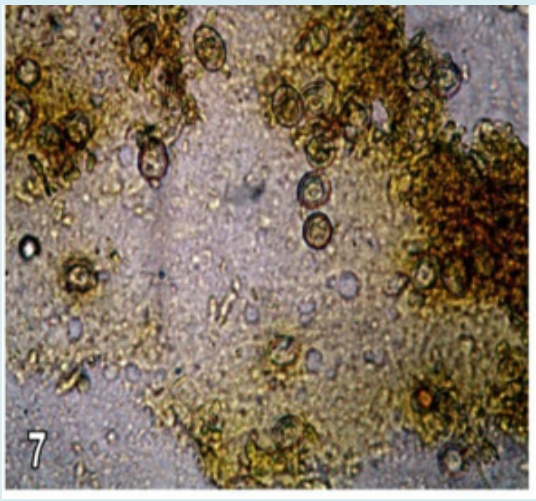

Figure 7: Caecal mucosal scraping showing heavy infection with oocysts from control chicks of group (5) at 38th day (X10).

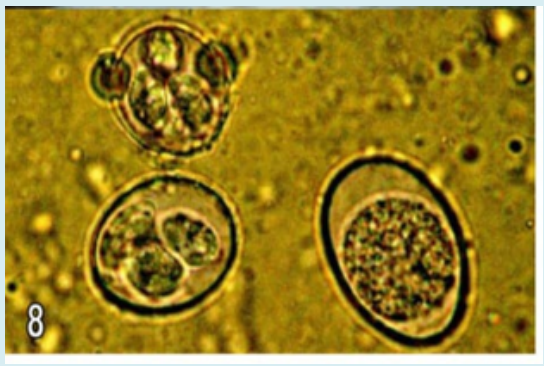

Figure 8: Faecal smear showing multiple oocysts and sporocysts from control chicks of group (5) at 36th day (X100).

Histopathological Finding

The histopathological examination of H& E stained sections of caeca from the different groups showed that there were multiple parasitic schizonts and oocysts in the mucosa and submucosa. There were invasion of the layers of the caecum by different inflammatory cells mainly lymphocytes and eosinophils. Small focal areas of necrosis in underlying connective tissue were seen. Small areas of hemorrhage and necrosis were seen separating the underlying connective tissue. The epithelium may contain sufficient parasitized cells that can produce degeneration of surrounding connective tissues. The caeca showed inflammation over 80% of the distal areas. The lumen is filled with blood and shedding of mucosa. Edema and necrosis are seen in muscularis mucosa and submucosal areas. Fibrosis is seen in the submucosal layer. Both asexual and sexual forms of the parasites develop beneath the nuclei of the epithelial cells. The number of schizont is increased and the degree of inflammation is severe. There was transmural inflammation affecting all layers of intestinal wall in group (4). While group (5) showed mild inflammatory infiltrate and extensive fibrosis. There were extensive invasion of the mucosa and muscularis mucosa by schizonts and inflammatory cells forming crypt abscess in group (1) Figures 9 & 10. There were moderate infection of schizont with the presence of Crypt abscess & parasitic granuloma in group (2) Figures 11 & 12. However, group (3) showed partial shedding of the mucosa, ulceration and infiltration by a large number of acute and chronic inflammatory cells (Figures 13-16).

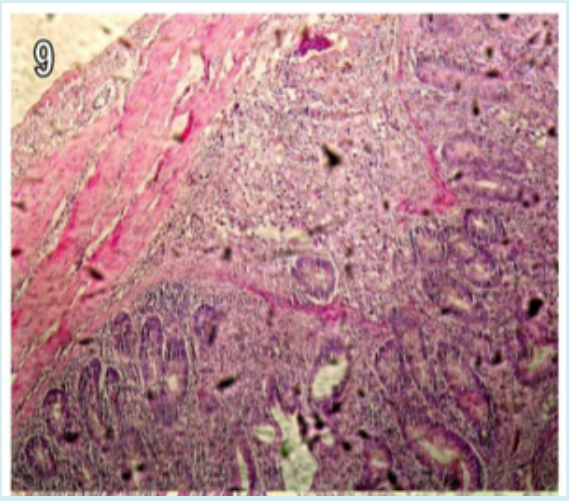

Figure 9: Section in the caecum from group (1) chicks showing transmural inflammation affecting all layers of the intestinal wall with submucosal invasion by schizonts (H&E X200).

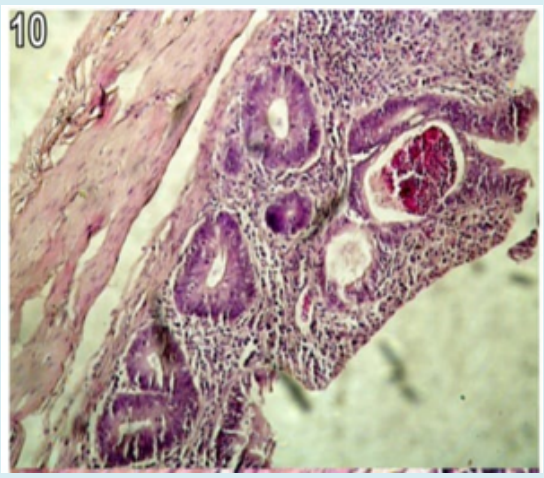

Figure 10: Section in the caecum from group (1) chicks showing acute and chronic inflammatory cells collected at the depth of the mucosal crypts forming a small crypt abscess (H&E X250).

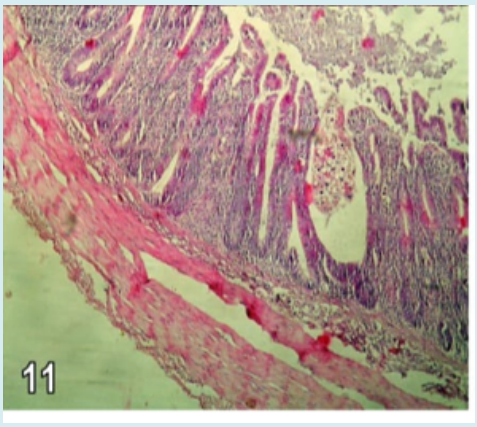

Figure 11: Section in the caecum from group (2) chicks showing one crypt abscess composed of Eimeria schizont and chronic inflammatory cells (H&E X250).

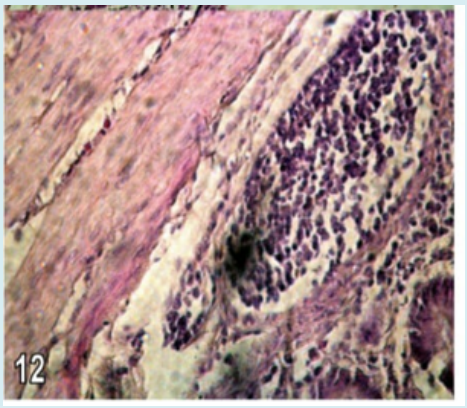

Figure 12: Section in the caecum from group (2) chicks showing inflammation of all layers of intestinal wall with parasitic granuloma composed of chronic inflammatory cells and fibrosis (H&E X 350).

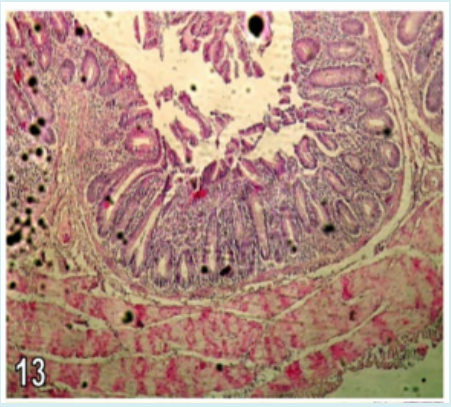

Figure 13: Section in the caecum from group (3) chicks showing partial shedding of the mucosa, ulceration and infiltration by a large number of acute and chronic inflammatory cells (H&E X250).

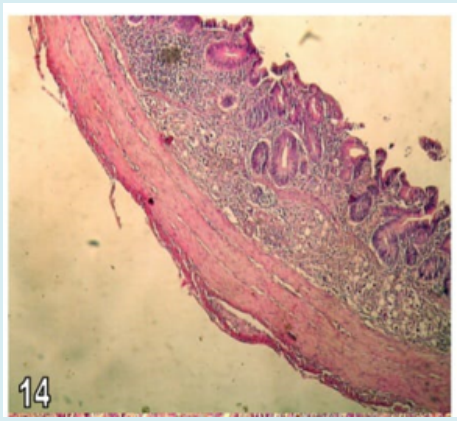

Figure 14: Section in the caecum from group (4) chicks showing submucosal invasion of schizont with transmural inflammation affecting all layers of intestinal wall (H&E X100).

Figure 15: Section in the caecum from group (4) chicks showing severe invasion of the mucosa by schizonts with marked fibrosis of the intestinal wall associated with regeneration of surface epithelium (H&E X100).

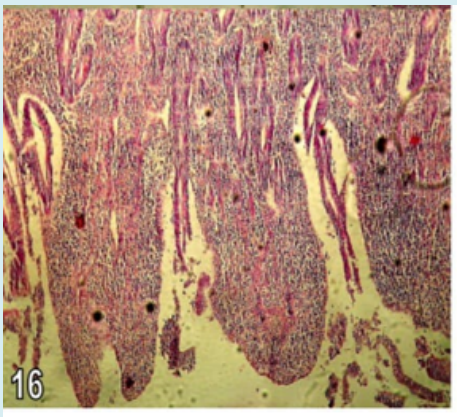

Figure 16: Section in the caecum from group (5) chicks showing loss of surface epithelium and intestinal shedding of the villi with marked inflammatory infiltrate and extensive fibrosis. (H&E X250).

Discussion

Coccidia are a group of intracellular opportunistic protozoa which contain different genera. They share general features in morphology, life cycle and pathogenicity. *Isospora* is a human intestinal coccidian closely related to members of the genus *Eimeria. Eimeria* causes disease in various domestic animals, fish and birds [1]. One of the main expenses facing poultry industry is the loss associated with poultry diseases, including costs of vaccination, prevention, treatment, reduction in weight gains, and mortality [22,23]. Coccidiosis is an economic and health problem in the poultry industry and can infect any type of poultry in any type of facility and its occurrence is worldwide [24].

This work was planned to study the effect of grinded *N. sativa* seeds and curcumin on experimental *E. tenella* infection in chicks. The present work showed that the results of total oocyst count in group (1) and group (3) were nearly the same count during the eight days post infection (p.i) which was statistically significant as compared to groups (4 & 5). However, group (2) had the lowest oocyst count as compared to the other test and control groups. These results were statistically significant. These results agreed with the work done by Cumming, [25]. However, Allen and Fetterer [14] showed no agreement with these results and stated that *N. sativa* had no therapeutic effect on *E. tenella*.

The present work showed that the total white blood cell count was decreased in group (1) (3,500 ± 270.7/µl), as compared to group (2) (20,500 ± 860.5/µl) at the age of 30th day old. However, significant differences were observed among the other groups. While at the age of 42 nd day, group (3) (69,600 ± 7,128/µl) showed high white blood cell count then group (2) (57,750 ± 5,687/µl) followed by group (4) (54,900 ±5,461/µl) as compared with control which was statistically highly significant.

These results agreed with the work done by Antony, et al. [26] who stated that the use of tumeric inclusion (0.5 and 1.0%) increased total leucocytic count. Also, Hashim, [9] found that the use of *Nigella sativa* (1000 ppm) inclusion increased the total WBC count in chickens due to improvement of the immune response in vaccinated chickens against coccidial infection.

The histopathological examination of caecal sections from the different groups revealed alteration of the epithelium, inflammatory cellular infiltration, abscess and fibrosis which differed in intensity among the different groups. Also, the number of schizonts and their location in the wall of the caecum varied widely. Group (2) showed mild infection with schizonts and there was cryptabscess and parasitic granuloma and the degree of penetration was superficial and slightly deep, there was sloughing of mucosa and regeneration in epithelial cells. Group (1) showed moderate number of schizonts in submucosal layer and crypt abscess which was comparable to group (2).

Group (3) showed partial shedding of the mucosa, ulceration and infiltration by a large number of acute and chronic inflammatory cells). Group (4) showed severe infection with schizonts and transmural inflammation affecting all layers of intestinal wall. Control group showed inflammatory infiltrate and extensive fibrosis, sloughing of mucosal cell with fibrin network entailing acute and chronic inflammatory cell. These results agreed with the work done by Schat, [27] who found sloughing of the intestinal lining exposing the lamina propria and Barker, [28] who found alteration of the villus structure.

Results from the present experiments indicated that the host response in group (1) differently than group (3) at the level of the caecal mucosa comparing to control. These results agreed with the work done by Rose [23]. In the present experiment, there was an increase in intestinal crypt depth. Crypt abscess and hyperplasia was found to be present in group (1) challenged birds and group (2). Which agreed with the work done by Fernando and McCraw [29].

Thus, it can be concluded that the use of *Nigella sativa* and curcumin are recommended for control of coccidiosis. Further studies on the role of N. sativa as an anticoccidial are recommended to confirm the results obtained by this work and to examine their role as human anticoccidial.

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