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Evaluation of nano-curcumin on experimentally induced coccidiosis in broiler chicks

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Abstract
The aim of the study was to evaluation of the effects of nano-curcumin and curcumin on experimentally induced coccidiosis in the broiler chicks. A total of 70 numbers of day old broiler birds weighing (40-50 g) were kept in deep litter system in a small group of 10 birds each. From 5th day of post infection, the birds showed some symptoms of diarrhoea, anorexia and weakness. From 6th day post infection, the birds started to show bloody diarrhoea, anorexia, weakness and death. A total of 12 numbers of dead birds of different groups are examined grossly as well as histopathological and confirmed for ceacal coccidiosis. The final body weight was recorded after 42 days of experiment. The bodyweight at the time of death was recorded as final bodyweight for the birds died prematurely. It was seen that Group 2 (Normal control) showed highest bodyweight (2260±52.64). Subsequently, Group 3(treated with amprolium) showed a bodyweight (2175.00 ± 49.75) which has no significant difference with Group 2. Group 6 (Traeted with nanocurcumin 300mg) showed the highest bodyweight among the experimental groups (2015.00 ± 43.02). Group 6 (treated with nano-curcumin) was showed better FCR value (1.78) among experimental groups. It was concluded that supplementation of nano-curcumin with the drinking water of broiler chicks may improves the body growth as well as fight against coccidiosis.

Keywords: Nano-curcumin, coccidiosis, broiler chicks, antioxidant

Introduction
A greater part of Indian economy is based on livestock industry and among them, the poultry industry is one of the fastest growing one. From 2011, the total production of broiler meat in India shifted from 2900000 MT (approx.) to 3900000MT (approx.) in 2015 with a growth rate of 7-8% per annum approximately (USADA, 2016). This is one of the newest industries in India as it was not commercialized till the last part of twentieth century. However, with expansion of these industries the chances of diseases are also increasing. Due to which, the poultry industry suffers a major lost every year. One of the most important as well as devastating diseases which is prevalent throughout the world and is found to be irrespective of season and environmental variation is poultry coccidiosis. Coccidia are the Apicomplexan protozoans belonging to the subclass Coccidia, family Eimeriidae and genus *Eimeria* (Finlay *et al*., 1993; Lillehoj & Lillehoj, 2000) [10, 17]. Several species of these intracellular protozoan parasites causes avian coccidiosis, those are; *Eimeria tenella*, *Eimeria necatrix*, *Eimeria brunetti*, *Eimeria praecox*, *Eimeria acervulina*, *Eimeria mitis*, and *Eimeria maxima* (Shirley, 1986) [22]. The site of predilections are different for different species. It causes reduced growth rate, impaired feed conversion ratio and main important is the mortality in poultry farm. There are various standard drugs found to be affective against coccidiosis like Amprolium, Diclazuril, Robenidine, Monensin, Sulphaquinoxaline, Salinomycin etc. But due to development of drug resistance and longer drug withdrawal period in modern commercialized farm and its higher price and non-availability in remote places for most of the small stock (backyard) farmers, it's becoming a rising problem which leads to a great loss in Indian poultry industry.
Various herbal complexes, e.g.: *Curcuma longa* (commonly known as Turmeric) are effective against poultry coccidiosis (Abbas *et al*., 2012) [1]. The active ingredient of turmeric is 'curcumin'. It is a phenolic compound and has antioxidative, anti-inflammatory and immune-modulatory properties (Allen *et al*., 1998) [4]. Abbas *et al*. (2010) [2] studied about its anticoccidial properties of *C. longa* and found effective in mild infection.

Curcumin is a bis-α-β-unsaturated β-diketone; under acidic and neutral conditions, the bis-keto form of the compound predominates, and at pH above 8, the enolate form is generally found (Sharma *et al*., 2004) [21]. Hence, at pH 3–7, it acts as an extraordinarily potent H-atom donor and above pH 8, it acts mainly as an electron donor, a mechanism more suitable to the scavenging or antioxidant properties of curcumin (Jovanovic *et al*., 2001) [12]. Curcumin is quite unstable at basic pH and degrades within 30 minutes. The normal form of curcumin is rapidly eliminated from the body and its organ penetration property is also very low. Tsai *et al*. (2011) [23] reported that the Nano form of curcumin (C-NPs) have significantly higher penetration and distribution rate, raised AUC (area under curve), $t_{1/2}$and MRT. The results helped in effective employing of curcumin in formulation of drug. The average particle size of the curcumin generally ranges in between 10 to 12 micrometer in diameter whereas in nanocurcumin ranges at about 330nm. The nano form of curcumin is easily dissolvable in water.

Considering the significance of poultry coccidiosis present in different regions of India the present study has been designed to evaluate the role of Nanocurcumin and curcumin separately in experimentally induced coccidiosis in broilers.

Materials and methods

The study was performed in accordance with the guidelines for the use and care of laboratory animals by Institutional Animal Ethical Committee (Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/15-16/355). A total of 70 numbers of Day old Broiler birds weighing (40-50 g) were procured from Local Hatchery. All the birds were kept in deep litter system in a small group of 10 birds each. Birds were fed with standard balanced ration and clean drinking water *ad libitum* and were vaccinated for Ranikhet Disease at 4th day with F-strain vaccine and maintained in a standard laboratory conditions (12:12 hour light/ dark cycle at ambient temperature ranging between 12-25^0 C). Identification of birds was done with picric acid marking on various parts of the body throughout the experiment during the entire study.

Preparation of Nano-curcumin by Evaporative Precipitation of Nanosuspension (EPN)

Curcumin was purchased from HIMED. All the reagents used were of analytical grade. N-Hexane (HPLC grade) and ethanol (99.5–99.8%, Absolute, GR Grade for analysis) were obtained from Merck. The deionized water was used in experiments.

As per Kakran *et al.*, (2012) [13] the solution of original curcumin was prepared in ethanol and then a nanosuspension was formed by quickly adding hexane (anti-solvent). Drug particles in the nanosuspension were obtained by quick evaporation of the solvent and anti-solvent, under vacuum using a rotary evaporator. This was followed by vacuum drying of the nano-particles to completely evaporate all the solvents. The drug concentrations used were 5, 10, 15 mg/mL and the solvent to antisolvent (SAS) ratios were varied from 1:10, 1:15 to 1:20 (v/v). For 20 mL of the drug solution, 200–400 mL hexane was used. Protocol of preparation of Nano-curcumin is as below:

A. 100 mg Curcumin (0.1gm) was added in 20ml Ethanol (5mg/ml) in a beaker.
B. 400 ml of Hexane was poured directly and quickly to the ethanolic solution. (SAS=1:20)
C. The solution was evaporated using a Rotary Evaporator.
D. The remaining residues were collected and lyophilized.
E. The newly obtained particles of curcumin were characterized for size by Zeta Sizer Machine, (IASST, Guwahati). The resultants were: Original size of curcumin- 4706 nm and newly formed nano-curcumin-140.5 nm

Acute Oral Toxicity

The study was carried out according to OECD (Organization of Economic Co-operation and Development) 420 guidelines. The nano-curcumin at different doses up to 2000 mg/kg was administered to the birds and was observed for behavioral changes, toxicity and mortality up to 24 hours. The birds were further observed for 7 days to record mortality. No mortality was observed in birds during the period and based on this study three doses of nano-curcumin (100 mg/kg, 200 mg/kg and 300 mg/kg body weight) was selected and used for evaluation of anticoccidial activity in broiler birds with ten birds in each group.

Collection and Sporulation of Oocyst

Feacal samples for coccidian oocysts were collected from commercial broiler farms during outbreak. The collected faecal samples were observed under microscope by direct method or using flotation technique. After detection and identification, the remaining samples were used for Sporulation. Sporulation was done by keeping the samples in 2.5% Potassium dichromate solution for 48 hours. Then Potassium dichromate was washed off from the feacal sample by centrifuging it with distilled water. Straining was done to the sample to remove the debris and feacal contents. The supernatant was removed and the remaining parts contained the sporulated oocyst. The sporulated oocysts were then counted by using McMaster Egg Counting method. 10,000-20,000 oocysts were being administered to each bird by oral route mixed with water (Jang *et al*., 2007 and Chen *et al*., 2008) [11, 9].

Administration of Drugs

Each bird was weighed on a balance to evaluate the dose of nano-curcumin, amprolium and curcumin.

Experimental Groups**:** Birds were randomly divided into seven groups with each of ten birds. A number of sporulated oocysts (10,000-20,000 sporulated oocysts per bird) were administered by oral route on 10th day of experiment (Jang *et al*., 2007 and Chen *et al*., 2008) [11, 9]. Amprolium (@ 2g/litter of water), three doses of nano-curcumin (100 mg/kg, 200 mg/kg, and 300 mg/kg body weight) and curcumin (300 mg/kg body weight) were administered orally daily upto 42nd day.

Group (I): Served as normal control, was administered normal wholesome water and feed for 42 days.

Group (II): Served as positive control, was administered 10,000-20,000 sporulated oocysts per bird at 10th day with normal wholesome water and feed for 42 days.

Group (III): Was administered 10,000-20,000 sporulated oocysts per bird at 10th day with amprolium @ 2g/L of water and feed for 42 days.

Group (IV): Was administered 10,000-20,000 sporulated oocysts per bird at 10th day with nano-curcumin@ 100mg/Kg body weight mixed in water and feed for 42 days.

Group (V): Was administered 10,000-20,000 sporulated oocysts per bird at 10th day with nano-curcumin@ 200mg/Kg body weight mixed in water and feed for 42 days.

Group (VI): Was administered 10,000-20,000 sporulated oocysts per bird at 10th day with nano-curcumin@ 300mg/Kg body weight mixed in water and feed for 42 days.

Group (VII): Was administered 10,000-20,000 sporulated oocysts per bird at 10th day with Curcumin @ 300mg/Kg body weight mixed in water and feed for 42 days.

Body Weight Gain Record

The body weights of all the birds were taken on daily intervals with weight of the feed consumed per day. The variations in body weight and feed consumption among the groups were analyzed statistically to determine growth performance, FCR etc.

Feacal Oocysts Count

Feacal samples were collected and oocysts were counted form 6th day (post infection onwards) i.e. from 16th day. Collection and oocysts counting were done by Mc master egg counting method on daily basis for one week i.e. upto 13th day post infection.

Lesion Scoring

Lesion scoring was performed by using the method described by Raman *et al*. (2011) [20]. Score was given from 0 to 4 on the basis of its gross lesion, degree of heamorrhage, thickness of ceacal wall etc. For *E. tenella,* the scores were

- **Score 1:** Scattered petechiae on the cecal serosal and mucosal surfaces. Little blood in the ceca. Thick cecal contents.
- **Score 2**: Petechiae on the cecal serosal and mucosal surfaces. Thick cecal contents containing blood or fibrin. Thickened wall, but presence of grooves.
- **Score 3**: Cecal wall greatly thickened grooves no longer visible. Absence of cecal contents replaced by blood or fibrin.
- **Score 4:** Distended and club-shaped ceca. The lumen of the distended ceca is all filled with blood (schizogony) or blood clots.

Statistical Analysis

The data of the present research work was analyzed by using students T-test for comparison between tests and control using standard statistical procedure.

Results and discussion

Clinical Findings

From 5th day post infection, the birds showed some symptoms of Diarrhoea, anorexia and weakness. From 6th day post infection, the birds started to show bloody diarrhoea, anorexia, weakness and death.

Table 1: Clinical findings from 5th day post infection (DPI)

Groups	Clinical Symptoms	Day Post Infection									Mortality	Bird Alive	Mortality Rate (%)
		5	6	7	8	9	10	11	12	13			
1	Weakness	+	+	+	+	+	+	+	+	+	10	0	100.0
	B. Diarrhoea		+	+	+	+	+	+	+	+			
	Mortality				+	+		+	+				
2	Weakness										0	10	0.0
	B. Diarrhoea												
	Mortality												
3	Weakness	+	+	+	+	+	+				0	10	0.0
	B. Diarrhoea		+	+	+	+							
	Mortality												
4	Weakness	+	+	+	+	+	+	+	+	+	1	9	10.0
	B. Diarrhoea		+	+	+	+	+	+	+				
	Mortality			+									
5	Weakness	+	+	+	+	+	+	+	+		1	9	10.0
	B. Diarrhoea		+	+	+	+	+						
	Mortality				+								
6	Weakness	+	+	+	+	+	+	+			0	10	0.0
	B. Diarrhoea		+	+	+	+	+						
	Mortality												
7	Weakness	+	+	+	+	+	+	+	+	+	1	9	10.0
	B. Diarrhoea		+	+	+	+	+	+					
	Mortality						+						

The result can be compared with Chandrakesan *et al*. (2009) [7], where they observed the clinical signs from sixth day onward. They administered sporulated oocysts at the dose rate of 30,000 sporulated oocysts per bird on 28th day and observed clinical symptoms like bloody diarrhea, weakness even mortality from 6th day post infection. Abbas *et al*. (2010) [2] found in their study that bloody diarrhea of almost all experimental groups, with the exception of the uninfected control group, was observed from the 4th to 6th day after challenge with *E. tenella*. In the groups treated with rations supplemented with salinomycin sodium, and 2 and 3% turmeric powder, the extent of bloody diarrhea was milder than that observed in other groups, which is completely supportive to our experimental findings. Kurkure *et al*. (2006) [16] reported about the clinical symptoms after challenge infection of coccidia where they found mild diarrhoea from 4th day post infection and severe diarrhea with blood tinge with mortality in birds from 6th day p.i. Comparison was similar with some other experiments like Kim *et al*. (2013) [15], Manafi (2011) [18] Abbas (2012) [1] etc., it can be concluded without any dispute that the clinical symptoms starts showing from 5th day onward, where as bloody diarrhea and mortality starts from sixth day post infection.

Mortality Rate

A total of 12 birds died during the experiment. All dead birds

are examined grossly as well as histopathologically and confirmed for ceacal coccidiosis. The negative control group (infected+untreated group) showed highest mortality (100%). Other groups showed less moratlity specially the curcumin and nanocucumin treated groups. The positive control group (treated with amprolium) and group 6 (treated with nanocurcumin 300mg/kg b.w.) showed no mortality.

Growth Performance

The growth performance was expressed in terms of bodyweight gain, Feed Conversion Ratio, Feed consumption rate etc.

Bodyweight Gain: The final body weight was recorded after 42 days of experiment. The bodyweight at the time of death was recorded as final bodyweight for the birds died prematurely. It was seen that Group 2 (Normal control) showed highest bodyweight (2260±52.64). Subsequently, Group 3(treated with amprolium) showed a bodyweight (2175.00 ± 49.75) which has no significant difference with Group 2. Group 6 (Treated with nano-curcumin 300mg) showed the highest bodyweight among the experimental groups (2015.00 ± 43.02). The results can be compared with the experiments of Abbas *et al.* (2012) [1], where they recorded the final bodyweight of the birds as 2298g in the uninfected untreated group (positive control), 2280g in the standard group, 1988g in 1% turmeric, 2023 in 2% turmeric, 2293 in 3% turmeric group. Here the results are slightly higher and it may be due to the larger dose rate of turmeric (30g per kg of feed). The results are also similar with Arczewska *et al*. (2012) [5], Manafi (2011) [18] and Christaki *et al*. (2004) [8] where they used some herbal extracts and the body weight gain was similar to that of nano-curcumin groups.

Table 2: Average bodyweight and feed conversion ratio of different groups of birds

Groups	Final Bodyweight(g)	Average Body Weight Gain (G)	Average Feed Intake (G)	FCR
1	598.00 ± 59.00^{g}	598	1334	2.23
2	2260.00 ± 52.64^{a}	2260	3708	1.64
3	2175.00 ± 49.75^{a}	2075	3465	1.67
4	1671.00 ± 41.40^{d}	1671	3250	1.95
5	1858.00 ± 41.90^{c}	1814	3285	1.81
6	2015.00 ± 43.02^{b}	1887	3376	1.78
7	1640.00 ± 47.92^{d}	1640	3158	1.92

*Values are expressed as Mean ± SE. significant level is ($p<0.01$). Means with the different superscript letter differs significantly

Feed Conversion Ratio (FCR): Group 2 (normal control) showed highest FCR (1.64). Subsequently, group 3 (treated with amprolium), group 6 (treated with nanocurcumin) showed 1.64 and 1.78, respectively. The other nano-curcumin treated groups showed 1.81 and 1.95. Curcumin treated group (group 10) showed an FCR of 1.92 and was better than nano-curcumin 100mg group. The results can be compared with Arczewska *et al*. (2011), where they reported the FCR after 42 days of experiments were 1.88 in normal control, 1.84 in herbal extract, 2.06 in negative control, 1.99 in standard and 1.93 in herbal extract group. The results were similar except significant difference between the normal groups and standard drugs. The variation in the results may be due to equal distribution in male and female chicks in their experiment, where as there was equal distribution in terms of sex here. The quality of chick and feed may also play a major role in the variation of the same. However, the nano-curcumin groups showed a better FCR than the curcumin group.

The results can be compared with Abbas *et al*. (2012) [1], where they found FCR 2.02, 2.25, 2.23, 2.00, 2.28 and 1.99, respectively. The bodyweight gain was slightly better in their study but in terms of FCR, nano-curcumin groups showed a significantly better result than that of turmeric 1%, 2% and 3% groups. Christaki *et al*. (2004) [8] also reported some common findings in terms of FCR. They reported 1.66 in normal group, 1.75 and 1.76 in Apacox groups and 1.64 in Lasalosid group. The value of nanocurcumin 300mg (1.78) group can be comapared with Apacox groups, which is an herbal anticoccidial agent available in market.

Estimation of Faecal Oocysts

Faecal Oocysts Were Counted Form Day 6 Post Infection. The Normal Control Group (Group 2) Showed No Faecal Oocysts. The Negative Control Group (Group 1) Showed The Highest Oocysts Count Per Gram Of Faecal Sample (87.94 ± 34.27). The Oocysts Count Was Done Up To 10^{th} Day Post Infection. Group 3, 4, 5 And Group 6 (Amprolium, Nano-curcumin 100mg, Nano-curcumin 200mg, Nano-curcumin 300mg) showed no significant differences. The results can be compared with Arczewska *et al*. (2012) [5] where they found the highest count of oocysts on day 6 p.i. and the counting were going down in subsequent days. The faecal oocysts count was higher and it was not completely absent on the last day of count, where as in the present study, the oocysts count was less in all groups group in comparison to the former and was completely absent on the last day of count in case of Amprolium group, Nano-curcumin 200mg and Nano-curcumin 300mg. The results are similar with the findings of Abbas *et al*. (2010) [2], where the oocysts count was higher in day 7 p.i. except in the negative control group, where the oocysts was higher in day 6 p.i. it may be due to the effect of the drugs which were being fed from the first day of infection. So, the oocysts count was not highest earlier but later on it increased and decreased subsequently. Christaki *et al*. (2004) [8] also reported the similar results with Lasalocid and Apecox 1g/kg group where they found a significant drop in oocysts count from $2.2x10^{6}$ to $0.3x10^{6}$ and $7.3x10^{6}$ to $0.5x10^{6}$ in 13^{th} day p.i. subsequently. The effect of Nano-curcumin (100mg, 200mg and 300mg) and curcumin 300mg can be compared to those anticoccidial preparations.

Table 3: Faecal oocysts count of different groups (X1000 oocysts per gram)

Groups	Days Post Infection								Mean ± SE
	6	7	8	9	10	11	12	13	
1	244	202	140	82	31	2	1.5	1	87.94 ± 34.27^{a}
2	0	0	0	0	0	0	0	0	0
3	19	24	11	4	1.5	0	0	0	7.44 ± 3.36^{c}
4	76	84	42	21	4.2	1	1	0.5	28.71 ± 12.29^{abc}
5	43	56	37	16	3.5	1	1	0	19.68 ± 7.93^{bc}
6	37	39	18	7	2	1	0	0	13.00 ± 5.85^{bc}
7	115	94	49	21	5	1	1	1	35.87 ± 16.16^{abc}

***** Means with the different superscripts in a column differ significantly

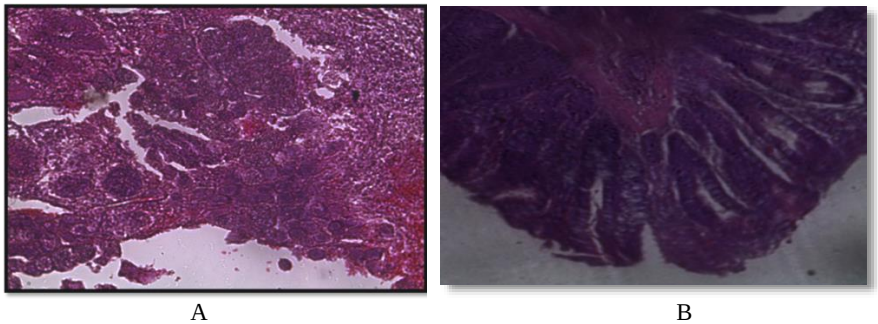
Lesion Score

Normal control group (Group 2) showed zero score where as the negative control group (group 1) showed highest scores (40). The next least score was seen in group 3 (amprolium) i.e. 2 and group 6 (nanocurcumin 300mg) i.e. 3. Among the treatment groups Group 7 (Curcumin) showed the highest score i.e. 14. In the groups of Nano-curcumin and curcumin, the lesion score (1.3, 0.9, 0.3 and 1.4) was found to be very less and better in comparison to the findings of Christaki *et al*. (2004) [8]. They reported to find the scores 2.1 and 2.6 in two different Apacox groups and 1.2 in Lasalocid groups. The result is far better in the present study in comparison to those anticoccidial preparations. It might be due to more number of birds (30 in a group) in their experiment and time of scoring of lesions i.e. at 7 days P.I., where as in this present study it was done in 32nd day P.I.(at 42nd day of experiment) to the survivors after the infection. Cejas *et al*. (2011) [6] also reported similar result and it may be concluded that Nano-curcumin @ 200mg and 300mg groups showed a better result in lesion score techniques where as curcumin group failed to show desired effect.

Fig 1: Different Pictures of Ceacum Used for Lesion Scoring

Histopathology

Histopathological study was done to support the lesion score technique. The histopathological slides were observed under microscope in 40X. The caecal tissue sections revealed various degrees of congetion, cellular infiltration, inflammatory changes, hyperplastic changes, necrosis heamorrhages, damage to the normal structure of the intestinal villi and most importantly the different developmental stages of *E. Tenella* (oocysts, merozoits, schizonts etc.). Group 1 (negative control group) showed various developmental stages of *E. tenella* under microscope. The tissue sections revealed damage to the tissue by sloughing off the mucosa and other lining epitheliums. Similar result was found in the experiments of Chandrakesan *et al*. (2009) [7], Jang *et al*. (2007) [11], Khan *et al*. (2008) [14], Kim *et al*. (2013) [15], Kurkure *et al*. (2006) [16] and Otoikhian *et al*. (2010) [19]. Nano-curcumin and curcumin groups showed some degrees of recovery and may be said effective as it revealed the absence of various developmental stages of coccidian and less amount of tissue damage in comparison to the tea waste extract groups. The Nano-curcumin 200mg and 300mg group showed same Histopathological structures as of the normal group.



A B

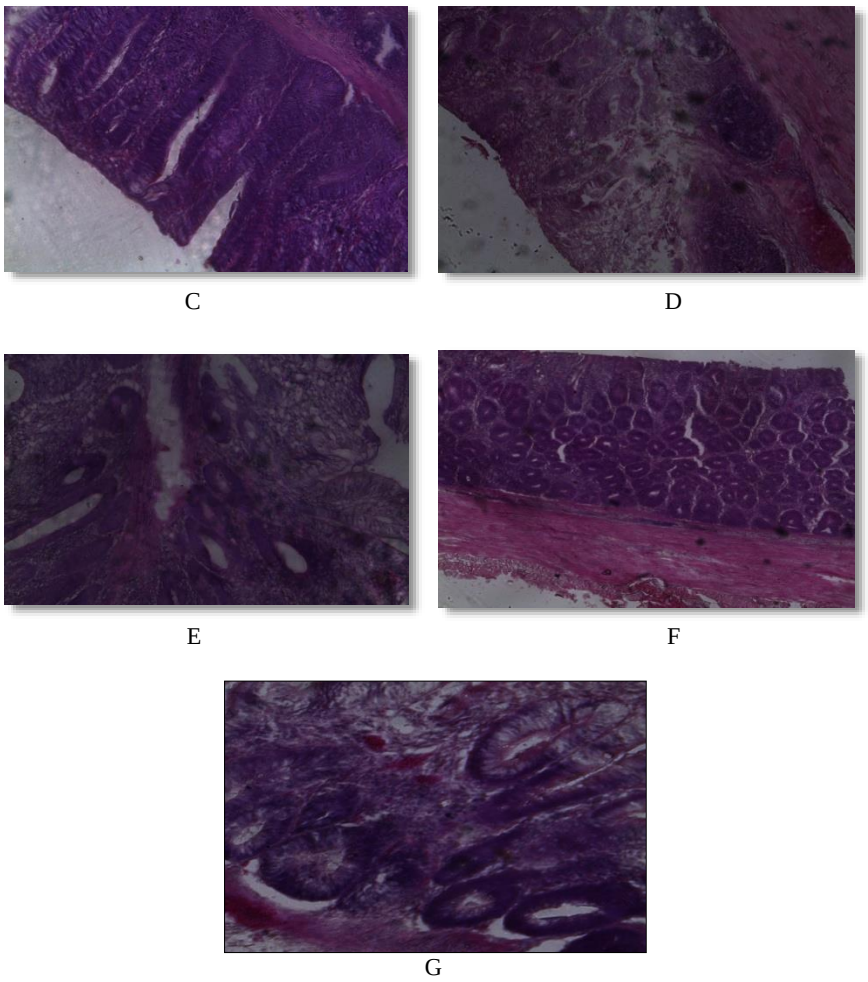


Fig 2: Histopathological Slides **A.** Group 1 (Infected+Untreated), **B.** Group 2 (No Infection+No Treatment), **C.** Group 3 (Treated With Amprolium), **D.** Group 4 (Treated With Nanocurcumin 100mg, **E.** Group 5 (Treated With Nanocurcumin 200 Mg), **F.** Group 6 (Treated With Nanocurcumin 300mg), **G.** Group 7 (Treated With Curcumin 300 Mg)

Table 4: Histopathological Findings of Different Experimental Groups

Groups	1	2	3	4	5	6	7
Presence of oocyst	+	-	-	-	-	-	-
Developmental stages of the protozoa	+	-	-	-	-	-	-
Sloughing off mucosal layers	+	-	-	+	+	-	+
Tissue damages	+	-	-	+	-	-	+
Haemorrhage with congestive spots	+	-	-	+	+	-	+
Necrotic lesions	+	-	-	+	-	-	+

Conclusion

The conclusions could be drawn from the present study as Nano-curcumin was found to be effective in experimentally induced caecal coccidiosis in broiler chicks and Nano-curcumin showed dose dependent effect.

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