



Anticoccidial properties of micronized curcumin against *Eimeria tenella* in experimentally infected broiler chickens

Abstract

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In this study, the anticoccidial properties of micronized curcumin against *Eimeria tenella* in a comparison with the ordinary one were investigated. The experiment was conducted using 105 broiler chicks, divided into four groups; micronized curcumin (A), curcumin (B), infected untreated (C) and uninfected untreated (D). Group A and B were treated for seven successive days with micronized curcumin (with sodium alginate) and curcumin suspended in corn oil respectively. All Chicks were infected orally with *E. tenella* at the 22th days of age except the control negative one. Depending on the anticoccidial indices (relative weight gain, survival rate, lesion value and oocyst value), the micronized curcumin in treated group showed a higher protection than curcumin treated. As anti-oxidant, both of curcumin treated groups revealed a significant increase in glutathione (GSH), superoxide dismutase (SOD) and catalase comparing with the control infected untreated group at $p \leq 0.05$. In addition, significant decrease of malondialdehyde (MDA) concentration of treated groups comparing with infected untreated group was recorded ($p \leq 0.05$). Since curcumin had anti-inflammatory properties, TNF α and IL 10 β R were significantly decreased in curcumin treated in a comparison with the infected untreated groups at $p \leq 0.05$. In conclusion, the micronized curcumin has a marked role in restricting the adverse effect of *E. tenella* infection in broiler chickens better than curcumin alone.

Key words: *Eimeria tenella*, curcumin, micronized, anticoccidial indices.

1. Introduction

Eimeriosis is an important avian parasitic disease infecting the poultry, it is caused by protozoa of the genus *Eimeria* and its infection leads to digestive disorders, mortality and great economic losses. *Eimeria* drug resistance still remains an enormous obstacle; *E. tenella*, *E. acervulina* and *E. maxima* are relatively tolerant to ionophores (Li et al., 2004). Overcoming drug resistant may be achieved by investigation of new drugs and different methods of administration; however, this increases the cost in the poultry

industry (Youn and Noh, 2001). Moreover, the disadvantages of conventional vaccines have also forced the research for some new alternatives, which are environmental friendly, target specific, and inexpensive. Curcumin (*Curcuma longa*) is a yellow natural polyphenolic compound extracted from turmeric root. The active compound (coloring agent) diferuloylmethane is responsible for various pharmacological effects of curcumin. These include anticancer; anti-inflammatory and antioxidant properties (Subramanian et al., 1994).

Antiprotozoal activities have been described as antimalarial activity (Nagajyothi et al., 2012), anti leishmania spp (Das et al., 2008). Using of curcumin (0.05%) was effective in reducing upper- and mid-small-intestinal infections caused by *E. acervulina* and *E. maxima* (Allen et al., 1998).

Curcumin alone is highly lipophilic, so the insolubility in water at acidic or neutral pH and its incomplete absorption lead to poor bioavailability (Ajay et al., 2012). Instability of curcumin in working solution, degradation during sample preparation, or overexposure time (Tonnesen and Karlsen, 1985) and by light (Sasaki et al., 1998) were taken into account to take full advantage of curcumin against *C. parvum*. Micronization is the process of reducing the average diameter of a solid material's particles. Consequently, micronization of curcumin leads to improvement of its bioavailability and its protective effect. Usually, the term micronization means the produced particles are few micrometers in diameter (Hassanin et al., 2013).

The current work aims to evaluate the anticoccidial properties of curcumin loaded alginate microcapsules and compare this effect with ordinary curcumin against *E. tenella* experimentally infected broilers.

2. Materials and methods

2.1. Curcumin forms

2.1.1. Curcumin loaded alginate microsphere

Curcumin powder (extract of *Curcuma longa*) was brought from SEGMA Company. Micronized particles with sodium alginate were adapted from an emulsification method described by Wan et al. (1992). Briefly, an aqueous solution containing sodium alginate and hydroxypropyl-methylcellulose (HPMC) (9:1) (5.0% w:v) was dispersed in an iso-octane solution containing a lipophilic surfactant (Span 85) (2.0% w:w) by using a mechanical stirrer at 8000 rpm. In the case of curcumin loaded microspheres (Bayrak et al. 2008), 100 mg curcumin / ml oil was added in the aqueous solution containing sodium alginate. An aqueous solution containing a hydrophilic surfactant was then added and the emulsion was stirred for 15 min.

2.1.2. Curcumin suspended in corn oil

Curcumin powder was dissolved in corn oil at a concentration of 100 mg/1ml oil of suspension.

2.2. Parasite material

PCR molecularly identified *E. tenella* oocysts of previous work in our laboratory were used in this study. Preserved oocysts in 2.5% potassium dichromate were washed with phosphate buffered saline and subjected to McMaster counting technique to quantify the number of sporulated oocysts per ml of suspension (Ryley et al. 1976). The final concentration of suspension was adjusted as a lethal dose (60000-70000) sporulated oocysts per 1 ml of PBS and stored at 4 °C till used in the experimental infection.

2.3. Experimental design

A total of 105(1-day-old Cobb 97) industrial broiler chicks were purchased from Cairo Co. Ltd. Egypt. The chicks were reared under standard management conditions in the laboratory of Parasitology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt. The chicks were fed commercial withdrawal feed (without anti coccidial agent). The chicks were allowed to acclimate for 14 days before the initiation of any experimental procedures. All chicks were inoculated with the routine vaccination against ND, Gumburo and Avian influenza. At the 14th day of age, chicks were randomly divided into four groups A, B, C (each one of 30 chicks) and D of 15 chicks. Each group was allocated as three replicates each in a pen. Chicks were reared in wire cages; Group A is the micronized curcumin treated group (curcumin + corn oil + sodium alginate). Group B is a curcumin suspended in corn oil (Curcumin treated group). Group C is an infected untreated group (Control positive). Group D is uninfected untreated group (Control negative). Groups A and B were administered two ml (100mg/ml) of curcumin solution for each one Kg body weight daily (200 mg/ 1Kg body weight) for seven successive days (from the 15th till the 21th days of age) according to Sudjarwo (2005). Prior to the infection, chicks were examined to confirm they were free from coccidia infection. The infection was done at the 22th day of age for the groups A, B, and C, by 60000 -70 000 oocyst of *E. tenella* orally by rubber oral gage for each chick.

2.4. Evaluation parameters for effectiveness of curcumin forms in the protection against *E. tenella* infection

2.4.1. Bloody diarrhea score (Dropping score)

Diarrhea scores were recorded between the 4th and 7th days post infection according to Du and Hu (2004). It was graded (0-4) as described by Morehouse and Barron (1970).

2.4.2. Survival rate

Calculation of survival rate for each group depends on the number of live birds.

2.4.3. Daily oocyst shedding per gram feces and the oocyst value

Daily oocyst counting was done at 7th day post challenge till the day of slaughtering (till 12th day post challenge).

The oocyst value = (OPG in each treated group) / (OPG in group of infected untreated group) × 100.

2.4.4. Gross lesion score and percent of protection

The percentage of protection against lesion score was calculated by using the formula described by Singh and Gill (1976).

Protection % = 4 – mean lesion score / 4 (maximum expected score) × 100

Lesion score of the chickens from each group was investigated according to the method of Johnson and Reid (1970).

2.4.5. Body weight gain and relative weight gain

Chicks in each group were weighted on the day of challenge (infection) (at 22th day of age), then at the 5th, 7th and 9th and at the end of experiment at 12th day post infection (PI). Weight gain of the chickens in each group was determined by subtracting the body weight of the chickens at the time of challenge from the body weight at the end of the experiments (Pinardet al., 1998). The percentage increase in body weight gains (piBWG) was calculated $\text{piBWG} = (\text{body weight after infection} - \text{body weight before infection}) / (\text{body weight before infection}) \times 100\%$. The relative ratio of body weight gains (rBWG) was calculated for each group according to the following equation:

$$\text{rBWG} = (\text{piBWG in each group}) / (\text{piBWG in group of uninfected untreated group} - \text{piBWG in group of infected untreated group}) \times 100\% \text{ (Ma et al., 2011).}$$

2.4.6. Anticoccidial indices (ACI)

The anticoccidial index was calculated by using the formula as described by Ma et al. (2011).

$$\text{ACI} = (\text{relative weight gain} + \text{survival rate}) - (\text{lesion value} + \text{oocyst value}).$$

An ACI > 180 indicated excellent activity, from 160 to 179 indicated moderate activity, from 120 to 159 indicated limited activity and <120 indicated inefficacy (Fei et al., 2013).

2.5. Biochemical parameters:

2.5.1. Oxidant/antioxidant status in tissues

Intestinal tissues were weighted and homogenized in ten volumes of ice-cold phosphate buffer saline (PH: 7) by using Homogenizer until a uniform suspension was obtained. The homogenate was centrifuged at 4000 rpm for 30 min at 4°C to separate supernatant from cellular debris. The supernatant was then used for the estimation of glutathione (GSH), malondialdehyde (MDA), superoxide Dismutase (SOD) and catalase enzymes in tissue according to Beutler et al. (1963); Satoh (1978); Nishikimi et al. (1972) and Aebi (1984) respectively.

2.5.2. Tumor necrosis factor alpha (TNF α) and IL 10 β

TNF α is measured by using Cayman ELA kits (Cyman Chemical Company, Ann. Arbor, MI, USA) according to the method described by (Grassi et al. 1991). Gene expression of IL 10 β receptor was performed on intestinal tissue homogenates by Real time quantitative polymerase chain reaction (qPCR) according to the methods described by Hogrefe (2002). The RNA extraction kit was supplied by promega (USA). The extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene, USA). The used primers of IL 10 receptor β gene are: forward primer (5 to 3) ATCTGTACCGATTACCAAG and Reverse primer (5 to 3) TTTAGAGTCCTGATGGTGCT. Its gene bank accession number is NM_204857.1

2.6. Statistical analysis

The results were analysed using the statistical SAS program SAS Institute (2002) and graph pad prism 6 (<http://www.graphpad.com/scientific-software/prism/>). The parametric data were analysed using the General Linear Models (GLM) procedure for analysis of variance. But the score results were subjected to nonparametric one-way (NPAR1WAY) analysis to calculate the differences between different treatment groups. For this the Kruskal-Wallis-Test for unpaired observations was used. For post hoc calculation of pair-wise differences between two different groups, the Wilcoxon two-sample test for unpaired observations within procedure NPAR1WAY was used. Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Bloody diarrhea score

All infected groups showed bloody diarrhea, but it was more significant in the infected untreated group (C) than the micronized group (A) at $p \leq 0.05$. While, there was non-significant difference between the curcumin treated (B) and the infected untreated groups (Fig 1).

3.2. Survival rate

The survival rate was 86, 66, 33 and 100% in group A, B, C and D respectively.

3.3. Oocyst shedding per gram feces (OPG)

Total oocyst shedding per gram feces showed a significant decrease between treated groups (A and B) and the control infected untreated one ($p \leq 0.05$) (Fig 2).

3.4. Gross lesion score and percentage of protection

There is a significant difference ($p \leq 0.05$) between the treated groups (A and B) and the control infected untreated. There was no difference in gross caecal lesion score between the treated groups (A and B). (Fig 3) and plate (1). Protection percentage against caecal lesions was the highest in micronized curcumin (60%) followed by curcumin (35.71%). The mean caecal lesions score value was significantly lower in micronized curcumin and curcumin treated groups than infected untreated groups ($p < 0.05$) (Table 1).

3.5. Body weight and body weight gain

A significant increase in body weight gain in the treated groups was reported in a comparison with the control infected untreated one. There was no difference in between treated groups. In addition, a significant difference between the control uninfected untreated group and the treated groups was reported (Fig 4) (Table 1).

3.6. Anticoccidial indices (ACI)

The calculated anticoccidial indices were 199.76 and 190.94 in micronized curcumin and curcumin treated groups respectively. The lowest anticoccidial index was 70.04 in infected untreated group. (Table 1), Fig 5.

3.7. Biochemical parameters

Curcumin treated groups showed significant increase in glutathione (GSH), superoxide Dismutase (SOD) and catalase in a comparison with the control infected untreated group at $p \leq 0.05$. A significant decrease of MDA concentration of curcumin treated groups in a comparison with the infected untreated group was recorded ($p \leq 0.05$). There were non-significant differences in the values of these enzymes in micronized curcumin, curcumin treated groups and uninfected untreated one. (Table 2).

3.8. TNF α value and IL 10 β receptor gene expression

The value of TNF α and IL 10 β in the treated groups were significantly lower than that of infected untreated group. There is non-significant increase of TNF α and IL 10 β in the micronized curcumin and uninfected untreated group (Table 2).

4. Discussion

The efficacy of anticoccidial compounds are based mainly on the anticoccidial indices; survival rate, lesion value, oocyst value and relative weight gain (Shah et al., 2009; Ma et al., 2011).

The survival rate recorded the highest rate in the curcumin treated groups compared with the infected untreated group. This finding supported by Deyab and Laji (2007), who recorded a high survival rate by *Curcuma longa* treatment in the ration against *E. tenella*.

Regarding the oocysts shedding, micronized curcumin and curcumin treated groups showed a significantly lower oocyst count than infected untreated one ($p < 0.05$). The same results were reported by Abbas et al. (2010); Lee et al. (2010) and Kim et al. (2013) when they used *C. longa* as dietary supplementation in poultry feed. Decreasing the oocyst count in treating by curcumin may be due to its ability to kill extracellular stages of intestinal protozoa (Khalafalla et al. 2011).

The weight gain was significantly ($P < 0.05$) higher in treated groups compared with the infected untreated one. Destruction of the intestinal tissue decrease feed conversion rate and the weight gain. Therefore, improving and maintenance of the weight gain of the chickens in curcumin treated groups, may referred to its direct cytotoxic activity against the *E. tenella* sporozoites and minimizing their invasion into the intestinal tissue (Khalafalla et al., 2011). This result was reported by Abbas et al. (2010); Lee et al. (2010); Kim et al. (2013).

Concerning the lesion value; there is irreversible relationship between the mean of this value and the protection rate. Micronized curcumin treated group achieved the lowest mean caecal lesion score values (highest protection rate). This may be due to the anti-inflammatory properties of curcumin. Invasion and intracellular development stages of *Eimeria* protozoan in the gut are associated with the induction of local inflammatory

response (Hong et al., 2006a, c); the latter was stimulated by the curcumin which lead to lesser gut damage according to Singh and Aggarwal (1995); Abe et al. 1999; Jagetia and Aggarwal 2007; Jurenka 2009.

Referring to the anti-oxidant effect, there was a significant increase in glutathione (GSH), superoxide Dismutase (SOD) and catalase with control infected untreated group at $P \leq 0.05$. However, a significant decrease of MDA concentration of curcumin treated groups with the infected untreated group was estimated ($P \leq 0.05$). The increased activities of GSH and SOD, in addition to, decreased MDA contents in the tissue, confirmed the antioxidant protective effect of curcumin (Huang et al. 1998). The antioxidant activity of curcumin was based on protecting the cell membrane through inhibition of lipid peroxidation (Khopde.1999). Increasing the GSH level to a great extent provides evidence for the involvement of GSH in the antiulcer activity of curcumin (Sudjarwo 2005).

The anti-inflammatory mediators, TNF α and IL 10 β in the micronized curcumin and curcumin treated groups were significantly lower than of the infected untreated group. Besides, there is non-significant increase of TNF α and IL 10 β in the micronized curcumin and uninfected untreated groups. In avian coccidiosis, several proinflammatory and T cell helper type 1 cytokines and chemokines (interferon- γ , IL-1 β , lipopolysaccharide-induced TNF alpha factor, IL-6, IL-8, and IL-10) were elevated in the chicken gut following infections with *E. tenella* (Hong et al. 2006a), and these soluble mediators of inflammation have been proposed to be responsible, in part, for intestinal damage during coccidiosis (Hong et al. 2006b). Administration of curcumin leads to a significant decrease of IL10 β R. The same finding was reported by Kim et al. (2013). The curcumin has activities as inhibitors of inflammation (Araujo and Leon 2001). The anti-inflammatory effects of curcumin are mediated through its ability to suppress the expression of proinflammatory cytokines, including tumor necrosis factor- α and interleukins (Jagetia and Aggarwal 2007).

The efficacy of micronized curcumin as anti coccidial agent reducing the mortality rate, lesion values and oocyst shedding, anti-oxidant and anti-inflammatory was better than curcumin alone. The latter is highly lipophilic, so the insolubility in water at acidic or neutral pH and its incomplete absorption lead to poor bioavailability (Ajay et al. 2012). Consequently, micronization of curcumin leads to improvement of its bioavailability and its protective effect. Bounding curcumin to phospholipids blended with turmeric essential oil and then micronized absorbed 8-10X (times) more effectively than Curcumin (Terry 2010).

From this work we concluded that the micronization of curcumin leads to improvement of its bioavailability and its protective effect against coccidian infection in broilers.

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6. Statement of Animal Rights

Ethical approval was obtained from a committee of Research, Publication and Ethics of the Faculty of Veterinary Medicine, Beni-Suef University.

8. Conflict of Interest Statement

There is no conflict of interest for this work

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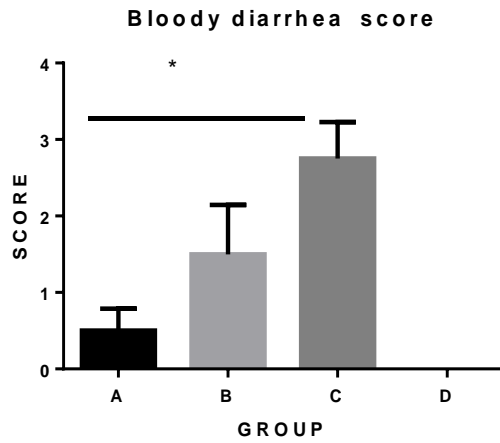


Fig 1. Bloody diarrhea scores of different groups.
* Superscript letters mean a significant difference between group A and C at $P < 0.05$.

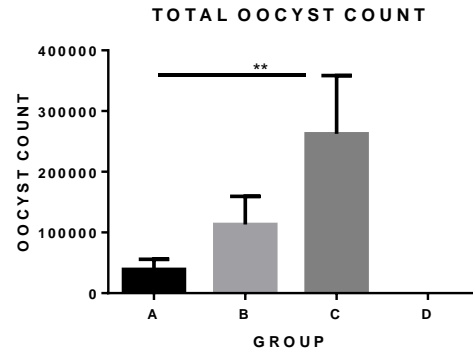


Fig 2. Total oocysts count.
* Superscript letters mean a significant difference between curcumin treated groups and group C at $p < 0.05$.

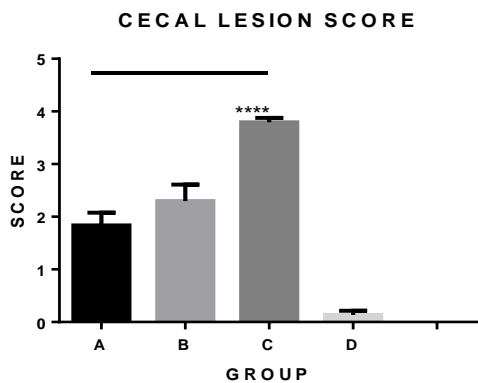


Fig (3). Cecal lesion score.
* Superscript letters mean a significant difference between curcumin treated groups and group C at $p < 0.05$.



Plate 1: Cecal lesion score.
1-Uninfected untreated control group (D)
2-Micronized curcumin group (A)
3- Curcumin treated group (B)
4-Infected untreated group (C)
5- & 6 – Bloody cecal core.

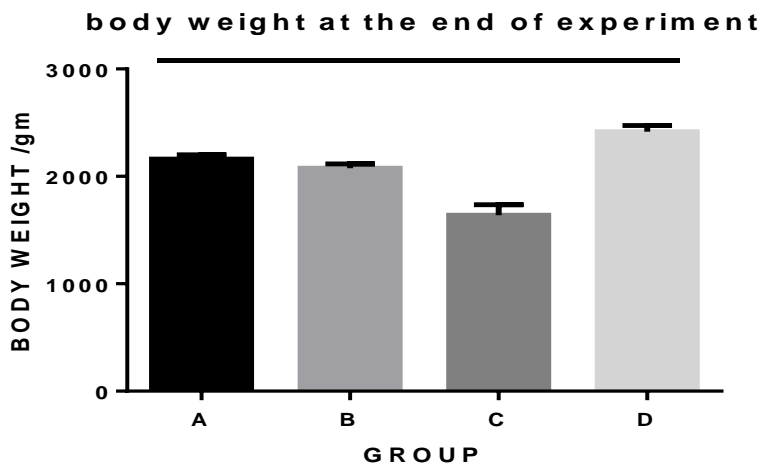


Fig 4. Body weight at the end of the experiment. A significant difference between curcumin treated groups and C at $p < 0.05$.

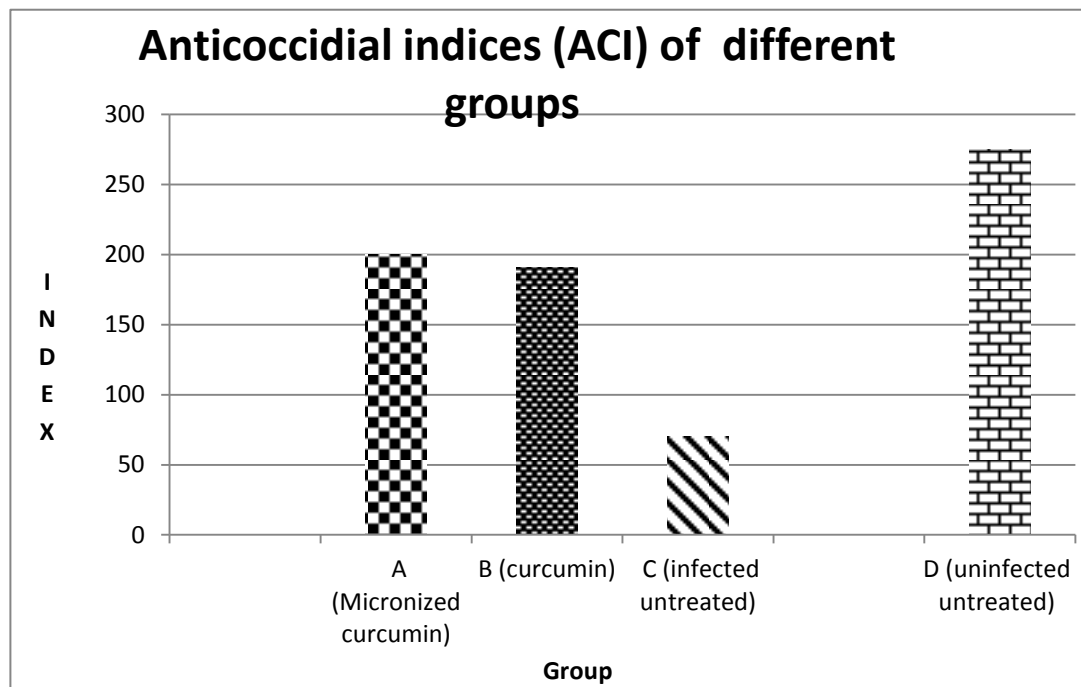


Fig5.The micronized curcumin and curcumin treated groups showed excellent anticoccidial efficacy (ACI >180).

Table 1. Calculated anticoccidial indices (ACI) in different treated groups

Group	Relative rate of wt. gain	Survival Rate	Lesion Value	Total Oocyst value	ACI
Group A (Micronized curcumin)	239.30	0.86	1.60	38.8 %	199.76
Group B (Curcumin)	236.00	0.66	2.57	43.15 %	190.94
Group C (Infected untreated)	173.37	0.33	3.66	100 %	70.04
Group D (Uninfected untreated)	273.78	1.00	0.00	0.00 %	274.78

An ACI > 180 indicated excellent anticoccidial activity, from 160 to 179 indicated moderate activity, from 120 to 159 indicated limited activity and <120 indicated inefficacy.

Table 2. The readings of four enzymes, TNF α and IL 10 R β values in different groups

Parameter	MDA (mg/ml)	GSH concentration (mg/ml)	Catalase (U/L)	SOD (U/L)	TNF α (pg/gm homogenate)	IL 10 (pg/tissue)	R β (mg)
Groups	mean \pm SD	mean \pm SD	mean \pm SD	mean \pm SD			
Group A (Micronized curcumin)	40.69 \pm 1.79 ^a	46.13 \pm 3.17 ^a	2.36 \pm 0.18 ^a	70.01 \pm 1.28 ^a	18.71 \pm 0.64 ^a	40.8 \pm 2.11 ^a	
Group B (Curcumin)	39.56 \pm 1.28 ^a	43.56 \pm 2.6 ^a	2.29 \pm 0.15 ^a	69.6 \pm 1.57 ^a	29.81 \pm 2.36 ^c	52.46 \pm 1.07 ^c	
Group C (Infected untreated)	62.74 \pm 2.34 ^b	23.8 \pm 2.35 ^b	0.90 \pm 0.04 ^b	38.4 \pm 1.72 ^b	57.26 \pm 2.87 ^b	65.22 \pm 2.01 ^b	
Group D (Uninfected untreated)	38.51 \pm 1.41 ^a	48.17 \pm 2.26 ^a	2.47 \pm 0.12 ^a	72.1 \pm 3.6 ^a	12.55 \pm 0.66 ^a	34.21 \pm 1.27 ^a	

Results are expressed as mean values \pm SD.

Different superscript letters mean a significant difference as compared to normal group at $P < 0.05$ in the same column.

خصائص النانو كوركوم كمضاد للايميريا تينلا في دجاج اللحم المعدى تجريبيا

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فى هذه الدراسة تم مقارنة النانو كوركوم والكوركوم العادى كمضاد للايميريا تينلا. تم اجراء التجربة على عدد 105 طائر من دجاج اللحم التى قسمت الى اربعة مجاميع (ا- ب- ج- د). كلا من مجموعة أ و ب تم اعطائهم النانو كوركوم والكوركوم العادى لمدة سبعة ايام متتالية بينما مجموعة ج مصابة وغير معالجة و مجموعة د ضابطة سلبية غير معالجة غير معدية. تم عدوى كل المجاميع ماعدا مجموعة د على عمر 22 يوم. اعتمادا على معدل زيادة الوزن- الحياة- الضرر النسيجي- اكياس الايميريا اظهر النانو كوركوم كفاءة اعلى من الكوركوم العادى. نظرا لان الكوركوم له خصائص ضد الاكسدة وجد ان الجلوتاثيون- السوبر اوكسيد والكتاليز اعلى من المجموعة المصابة وغير معالجة. بينما انخفض المألون الدهيد مقارنة بالمجموعة ج. مما سبق يتضح النانو كوركوم له تاثير مضاد للايميريا تينلا اعلى من الكوركوم العادى.