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Effect of nano-capsule of black cumin (*Nigella sativa* L.) seeds extract on broiler performance, intestinal micro flora and immune organs index

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Abstract

This study aimed to use the chitosan nanoparticles as a carrier of the black cumin seed extract (BCSE) to enhance the stability of the bioactive compounds in hard conditions and their effects on the growth performance, intestinal health and the immune organs index in broiler. Method used in the preparation of the chitosan nanoparticles was by the ionic gelation followed by oven drying at 40 C°. A total of 135 one day old broilers Ross (308) were divided into 3 treatments groups with 3 replicate per each group and 15 chicks per each replicate. The treatments were G1 (Control): basal diet without additives, G2; basal diet + 0.5% black cumin seed extract (BCSE), G3: basal diet + 0.5% black cumin seed extract nanoparticles. Results revealed that the adding of the BCSEN to diets improved (P<0.05) body weight, body weight gain, and feed conversion ratio as compared to the G1 and G2 during the periods from 1-21 and 22-42 days, with the exception of food intake, where there were non-significant differences between the G2 and G3. Also the BCSEN in broiler diets decreased (P<0.05) total aerobic bacterial and coliform bacterial counts as compared to the G1 and G2. The results also showed that both BCSE and BCSEN caused a significant increase (P<0.05) in the relative weight index of the immune organs (Spleen, Thymus, and Bursa of Fabricus) as compared to the G1, but the best results were achieved in the G3, In comparison with the G2. The nano capsulation of the black cumin seed extract (G3) could profoundly improve the growth performance, intestinal health, and the immune organs index, and can therefore be used instead of direct usage of the extract in poultry diet.

Keywords: Chitosan, Black Cumin, Nanoparticles, Broiler.

Introduction

Antibiotics were the major growth promoters in poultry. However, the use of Antibiotics is critical due to emersion resistance pathogens and their residues in tissues (Schwarz *et.al*, 2001). Consequently, many countries had banned the use of antibiotics in animal feed (Toghyani *etal,* 2010) and the scientists searched for the alternative natural substances that stimulate animal growth without hazardous effects on consumers health (Tamoor et al, 2014) . One of the alternatives is additions of medicinal plants or their extracts (Barreto *etal*, 2008) which proving more beneficial because of their anti-effects to different pathogens (Elgayyar *etal*, 2001). Also, medicinal plants possess a significant role as antioxidant, immunological–stimulant and stimulating effects on the animal digestive system (Jamroz and Kamel, 2002). As an example Nigella Sativa (Black Cumin) a plant belong to the family Ranunculaceae is known since ancient in flock medicine for its medicinal properties (Cheikh – Rouhou *etal*, 2007).

The seeds of black cumin enriched with volatile oil (0.5–1.6 %), fixed oil (35.6 – 41.6 %) and protein (22.7 %), also contain variety pharmacologically active substances such as alkaioids, thymoquinone, dithymoquinone, carvacrol, nigellicine – N – oxide, nigellidine and α-hedrin (AL- Gaby, 1998). So the seeds appear to be a promising growth stimulator in improve production and immunological status for poultry (AL-Homidan *et al*, 2002; Denli *et al*, 2004).

Actually, there are conflicting results regarding the using black cumin seed or their extract in poultry diets, many researchers had found encouraging results concerning to growth performance, food intake and efficiency, carcass traits, egg production and immunity were improved by incorporating black cumin seeds or their extract in the broiler or laying hens diets (Aydin *etal*, 2008; Toghyani *et al*, 2010; Ismail, 2011). However, contradictory results were reported by Hermes *et al* (2009); Nasir and Graskorn (2010), they found no significant effect or significant decrease in production and immunological traits due to the inclusion of black cumin seeds in the diets. These contradictions due to that the bioactive compounds in the medicinal plants and their extracts are susceptible to damage by harsh environmental conditions (light, temperature, oxidation) or by elevated PH in gastro intestinal tract render these compounds ineffective (Dudhani and Kosaraju, 2010; Aruna *et al*, 2011).

Recently, nanoparticles has been increasingly used in protecting and delivery many compounds such as enzymes, proteins, vitamins, drugs and other materials leading to increase stability, enhance absorption and bioavailability (Lee *etal*, 2010).

Most nanoparticles prepared from polymers , among them chitosan, a cellulose – like polymer located in the exoskeletons of arthropods such as crabs , shrimps , lobsters and insects (Fruda, 1983), is one of the most extensively studied , this is because chitosan possess some ideal properties of polymeric carriers for nanoparticles such as biocompatibility, biodegradability, no toxicity and low cost (Hejazi and Amiji, 2003). These properties render chitosan a very attractive material as a drug or effective material delivery carrier (Le Houx and Grondian, 1993).

DNA zyme and Si RNA is used in gene therapy by encapsulating it in chitosan nanoparticles (Tan *etal*, 2009), bio-adhensive nanoparticles of fungal chitosan are used for oral DNA delivery (Plapied *et al*, 2010).

According to the our information , there is no previous studies regarding to using of nanoparticles as a carrier material of black cumin seed extract to protect its effective compounds from damage in harsh conditions. So, the present study was conducted to detect whether the black cumin extract efficacy can be enhanced by its delivery through non – toxic chitosan nanoparticles and its effect in the production traits, intestinal bacterial content and immune organs index in broiler.

Materials and Methods

Preparation the black cumin seed extract: The Stoica et al (2013) method was used to preparation the black cumin seed extract with some modification, briefly, 20 gm. of seeds powder were immersed for 60 minutes in 100 ml of absolute ethanol : distiied water (v / v) . The mixture placed on magnetic stirrer for 40 minutes, centrifuged at 4000 rpm for 20 minutes. The residue was re-extracted by repeating the procedure that mentioned above. The supernatants were evaporated at 40° c under vacuum to remove the solvents, concentrated up to 20 % (w / v) of solid content and kept at 4o c until use.

Nano-capsulation of black cumin extract: Nano-capsulation of seed extract was done by ionic gelation method which described by Amir et al (2017) with some modification. Briefly , the chitosan solution prepared by adding 0.18 % (w / v) to distilled water containing 2 % acetic acid and 1.2 % Tween 80 . The solution was stirred with magnetic stirrer for 72 h with sonication every 24 h, then 20 % (w / v) of seed extract were added to the chitosan solution and stirred on magnetic stirrer at 4000 rpm for 20 min. The drop wise addition of 10 ml sodium Tripolyphosphate (STPP) solution (1 mg / ml) to a 25 ml CS solution (PH = 4) under constant stirring to formation CS–STPP nanoparticles loaded with BCSE . The mixture precipitated, filtered and oven dried at 45° c.

Characterization of The nanoparticles: The measurements of particles size was performed by dynamic light scattering technique, using the Nano Brook 90 plus particle Size Analyzer, and the Zeta Potential Size Analyzer was measured by using Nano Brook Zeta Potential Analyzer.

Animals, diets and management: This experiment was conducted to study the effect of nanoparticles of Black Cumin (Nigella Sativa L.) seed extract (BCSE) on broiler performance, intestinal micro flora and relative weight of immune organs.

The study lasted 42 days from 1st April to 12th May 2017 at the poultry farm of Animal Resources and Fisheries Center / Agricultural Researches Directorate / Ministry of Science and Technology.

A total of 135 one day – old chicks (Ross 308) with initial weight 41 ± 1 gm were used in this experiment. The birds randomly distributed to 3 equal groups (45 birds / group), each was divided to 3 replicates (15 birds / replicate). The experimental groups consisted of G1 as a control (basal diet without additions); G2 contained basal diet plus 0.5 % BCSE and G3 containing basal diet plus 0.5 % nanoparticles of BCSE.

A tow – phase diet was used in chick feed , a starter diet (22 % crud protein , 2926 K Cal / Kg) from 1 – 21 day and finisher diet (19 % crud protein , 3109 K cal / Kg) from 22 – 42 day .

The chicks were kept in floor pens (1×1.5 m^2) covered with shavings as litter material. Standard management practice (temperature, ventilation, and lighting) of commercial broiler production was applied; the birds were vaccinated against Infections Bronchitis, New Castle and Gumboro diseases according to the vaccines program in the broiler commercial farms.

Growth performance parameters: Average live Body weight (LBW), Body weight gain (BWG), Feed intake, and feed conversion ratio (FCR) were estimated for the starter phase (1 – 21 day), finisher phase (22 – 42 day) and the entire duration of the study (1 – 42 day) according to AL –

Zubaidi (1986).

Counting the Intestinal Micro flora: At the end of the experiment, 3 birds were selected from each replicate, slaughtered, the intestinal was extracted and kept refrigerated until transferred to the laboratory within 40 minutes to intestinal micro flora enumeration, with included Aerobic bacterial count, Coliform count and Lactobacilli count according to Harring and Mc Cane (1976).

Immune organ Index: In order to assess the immune organs index, at 42 day of experiment, 3 birds per cage were randomly chosen to evaluated the relative weight to Thymus, Spleen and Bursa. The relative weights of these organs were calculated as a percentage of live body weight was according to Chang – Song et al (2017) using the following formula:

RWO = WO / LBW × 100.

RWO = Relative weight of organ.

WO = weight of organ.

LBW = Live body weight of sample.

Statistical analysis: Data were subjected to analysis of variance (SAS, 2001) and significant means were separated by Duncan multiple rang test (1955).

Results and Discussion

It is noted from the table (1) that the average of particles size and zeta potential were 34.2 nm, 31.4 mv for the plain (csn) chitosan nanoparticles and 156.6 nm, 35.8 mv for the chitosan nanoparticles, with Black cumin extract (CS-BCE).

The reason for larger diameter size of CS-BCE nanoparticles is the result of swelling or aggregation of particles when dispersed in deionized water (Keawehaoon and Yoksan, 2011), nevertheless this size obtained are within the nanoscle, they should have a particle size less than 500nm (Quintanar *etal*, 1998).

The results of zeta potential to nanoparticles indicate the stability of the nanoparticles; wither for plain chitosan or the chitosan with the extract. (Couvreur *etal*, 2002) explained that the value of the zeta potential when were higher than 30 mv or less than -30 mv will help maintain the stability of the nanoparticles.

Table (1): The size and Zeta potential of the synthesis chitosan nanoparticle and chitosan nanoparticle with black cumin seed extract.

Sample	Average particle size(nm)	Zeta particle (mv)
Chitosan nanoparticle	84.2	31.4
Black cumin seed extract with chitosan nanoparticle	156.6	35.8

The results that represented in table (2) illustrated the effect of BCE (T2) and BCEN (T3) on broiler performance. The BCE n increased (P<0.05) the body weight (BW) and body weight gain (BWG) at the age 1-21 days, 22-42 days and 42 days compared with control group (T1) and BCE group (T2), we note that the T2 has a non-significant increase in these parameters compared to the T1 group.

Food intake did not significantly differ in the experimental groups at the period from 1-21 days, but both BCE and BCEN increased (P<0.05). Feed intake (FI) compared to the control group (T1) during the periods from 22-42 and 1-42 days, but it was not significantly different between the BCE and BCEN. All groups did not differ in Feed Conversion Ratio (FCR) at 21 days but supplementation of BCEN improved (P<0.05) FCR compared to BCE and control at the period 22-42 and 1-42 days, while it was not differences between T1 and T2 in terms of FCR.

The possible reason for the non-significant effect of BCE (T2) on the broiler performance is because that there are a limitations on the direct usage of the extract in poultry diet, that are related with harsh environmental conditions (temperature, light, humidity and extracting methods) which resulted in the oxidation and damage of the active compounds of the plant extract. On the other hand, these compounds may be degraded or digested by gastrointestinal juices or enzymes that render bioactive compounds ineffective (Amir *etal*, 2017). These results are consistent with (Ismail, 2011), who found no significant effect on broiler performance by the addition of BCE to diet.

So, to overcome these limitations, the delivery of plant extract through the chitosan nanoparticles as a vehicle is the better way to protect and improve the efficiency of bioactivity of the plant extract. Many studies reported that the addition of the turmeric, aloe, dill, and nettle extracts with chitosan nanoparticles was improve the broiler performance as compared to the direct addition of the plant extract to the diets (Sundari *etal*, 2014; Amir *etal*, 2017).

Table (2): effects of dietary black cumin seed extract or its nanoparticles supplementation on broiler performance.

Parameters	Day	Treatment			
		G1	G2	G3	SEM
Live body weight (g/bird)	1	41.2±1.4a	41.2±1.3a	41.3±1.1a	N.S
	21	639.5 ±11.35b	656.7±17.2b	687.9±14.1a	0.05
	42	1898.6±23.0 b	1950.7±29.3b	2073±18.7a	0.05
Body weight gain(g/bird)	1-21	598.3±11.5 a	615.5±9.6b	646.6±16.1a	0.05
	22-42	1259.1±29.7 b	1293.4±33.6b	1385.4±30.1a	0.05
	1-42	1857.4±22.2 b	1908.8±18.9b	2032.1±11.9a	0.05
Feed intake(g/bird)	1-21	1351±17.6 a	1350±15.1a	1345±19.2a	N.S
	22-42	2229±36.6 b	2293±41.1a	2273±39.3a	0.05
	1-42	3508±41.7 b	3643±81.1a	3618±43.7a	0.05
Feed conversion ratio	1-21	2.26±0.12 a	2.19±0.14a	2.07±0.8a	N.S
	22-42	1.77±0.01 a	1.77±0.9a	1.64±0.16b	0.05
	1-42	1.93±0.9 a	1.91±0.15a	1.78±0.13b	0.05

Mean in the same row with different letters differ significantly (p≤0.05).
SEM: Standard error of the mean, N.S: non-significant.
G1: Basal diet without additives (control).
G2: Basal diet + 0.5% black cumin seed extract.
G3: Basal diet + 0.5% black cumin seed extract nanoparticles.

This is agree with our results, because the BCE which in the Chitosan-STTP nanoparticles became more available to the birds in T3. This is was due to the chitosan was protected by STTP via ionic bonds, this will protect it from degradation in the presence of high acidity in the proventriculus stomach (Aranza *etal*, 2009), beside the nature of chitosan can open tight junction making it easier to absorb in the chicken small intestine (Sailaja *etal*, 2010). In this way, the bioactive compounds of the BCE will be transferred without exposure to the digestion or degradation in the gastrointestinal tract.

The possible reason of improve broiler performance in T3 compared to T1 and T2 may be related to the effective delivery of the active ingredients of BCE especially the essential oil by the chitosan nanoparticles. Many studies showed that the essential oils of BCS has a stimulate effect to the broiler digestive enzyme that improve the digestive of diet nutrients and feed efficiency which subsequently increasing the growth rate (Abu-Dieyah and Abu-Darwish, 2008; Ismail, 2011).

The results in table (3) showed that the dietary inclusion of BCSEN caused a significant (P<0.05) decrease in total aerobic bacteria and coliform bacteria with significant increase (p<0.05) in the numbers of lactobacilli bacteria in the intestine content of birds in G3 compared to G1 and G2. These results may be due to increase the bioavailability of the active compounds of the extract; especially the essential oil and thymoquinone by encapsulated them with chitosan nanoparticles.

Table (3): Effect of black cumin seed extract or encapsulated black cumin seed extract on intestine microbial populations(Log/========)

Parameters	G1	G2	G3	SEM
Total aerobic count	12.21±0.17 a	12.06±0.12 a	10.08±0.18 b	0.05
Coliform count	5.83±0.14 a	5.76±0.11 a	5.01±0.15 b	0.05
Lactobacilli count	3.67±0.17 a	3.78±0.16 b	4.69±0.13 a	0.05

Mean in the same row with different letters differ significantly (p≤0.05).
SEM: Standard error of the mean, N.S: non-significant.
G1: Basal diet without additives (control).
G2: Basal diet + 0.5% black cumin seed extract.
G3: Basal diet + 0.5% black cumin seed extract nanoparticles.

In agreement with the previous studies that showed that the essential oil and thymoquinone of the black cumin seed have a strong potential for reducing a different species of the pathogenic bacteria (Bolukbasi *etal*, 2009; Bourgou *etal*, 2010), furthermore, the chitosan has antibacterial activity against the gram-negative bacteria through the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes, which caused a changes in the particles of membrane permeability, thus provoke internal osmotic imbalance and consequently inhibit the growth of the microorganisms (Rejane *etal,* 2009). Thus, there will be an opportunity to multiply and increase the count of the beneficial bacteria such as lactobacilli in the intestine and inhibit colonization of the pathogenic bacteria (Guo *etal,* 2004; Rejane *etal,* 2009; Ismail; 2011).

We note from the results of table: 4 that the relative weight of the immune organs in G2 were significantly higher (P<0.05) than of those in the control group (G1). In fact, there were no previous studies showing the effect of the black cumin seed extract in relative weight of the immune index in broilers, but, Al-Beitawi *etal,* 2009 and Toghyani *etal,* 2010 reported that the addition of black cumin seed in the diet improved the weight of the lymphoid organs in the broilers, however, the mechanism is not clear. As well asm we noted from the same table (Table: 4) that the addition of the black cumin seed extract encapsulated with chitosan nanoparticles (G3) showed a better effects on the immune organs compared with the G2 and G1, and this may be due to the synergistic effect at both of the extract and chitosan.

Some studies suggested that the chitosan possesses antioxidant activity against free radicals and protecting the different body organs against damage (Guo *etal*, 2008), therefor, the chitosan may have played an important role in protecting the immune organs from damage by the free radicals and then improving its relative weights in the G3 compared to the G1 and G2.

The previous studies explained that the giving of a low concentration of dietary chitosan could increase the immune organs in broiler and ducks (Shi-bin *etal,* 2005; She-bin and Hong, 2012).

Table (4): Effect of black cumin seed extract and encapsulated black cumin seed extract on immune organ index of boiler (mean± SE).

Immune organ	G1	G2	G3	SEM
Bursa of fabricius	1.9±0.02 c	2.42±0.005 b	2.48±0.003 a	0.05
Thymus	3.53±0.04 c	4.40±0.002 b	4.51±0.001 a	0.05
Spleen	1.5±0.02 c	2.14±0.002 b	2.22±0.002 a	0.05

Mean in the same row with different letters differ significantly (p≤0.05).
SEM: Standard error of the mean, N.S: non-significant.
G1: Basal diet without additives (control).
G2: Basal diet + 0.5% black cumin seed extract.
G3: Basal diet + 0.5% black cumin seed extract nanoparticles.

Conclusions

We concluded from this study that the delivery of black cumin seed extract through chitosan nanoparticles as a carrier is a better way to improve the efficiency bioactive compounds of the extract and their effects on the growth performance, intestine health and the immune index in broiler.

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