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EFFECTS OF SPRAYING CEMELE PEPPER (*Capsicum annuum* L.) EXTRACTS ONTO HATCHING EGGS OF ATAK-S LAYER HENS ON HATCHING RESULTS, SERUM GLUTATHIONE AND MALONDIALDEHYDE LEVELS

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ABSTRACT

Alternative natural products have been used in hatching since chemicals used in the incubation of chicken eggs have toxic effects on the embryo, the practitioners and the environment. In this study with completely randomized design (CRD) the effects of a registered local pepper (Cemele) extract at different dosages in the incubation process of hatching eggs was examined. The chloride group disinfectant (T1), ethanol (T2), 2% pepper extract (T3), and 4% pepper extract (T4) were sprayed on to disinfect eggs before the incubation process. On the second, eighth, and eighteenth incubation days of the groups, the total mesophilic aerobic bacteria (TMAB) loads of the eggshell were examined, and the lowest load values were found at T4. In this study, the highest hatching efficiency (H) and the highest fertilised hatching rate (HF) were 87.52±2.88% and 81.66±1.44%, respectively, in T4. Early embryonic mortality (EEM) was not observed at T4, while the highest value was 7.11±1.35% at T2. The highest mid embryonic mortality (MEM) rate was 10.91±0.34% in T3 while the lowest rate was 7.11±1.35% in T4. The late embryonic mortality (LEM) rates were 14.22±2.69% in T2, 12.47±1.17% in T1, 12.66±2.69% in T3 and 5.30±0.17% in T4. The lowest malposition rate 8.92±0.01% and the lowest malformation rate 8.83±0.12% were observed in T4. At the end of this study, body weights, lengths, tona, and pasgar scores of the hatched chicks were calculated. The highest body weight and length values were observed as 43.57±1.37 g and 16.47±0.45 cm in T4 birds, respectively. The highest tona score was 98.10±1.27 in T4 and the lowest tona score was 93.65±1.22 in T1 birds. Pasgar score values of the chicks were 9.69±0.16 in T4, 9.38±0.17 in T2, 9.31±0.18 at T3 and 9.16±0.16 in T1, respectively. The MDA values of birds were 8.63±0.05, 0.70±0.10, 0.28±0.01 and 0.25±0.01 nmol mL^{-1} in T1, T2, T3 and T4, respectively while the highest GSH value was 32.39±0.66 μM in T4, and the lowest value 11.15±1.03 μM in T2. In conclusion, the use of Cemele pepper extract had positive results in the incubation process of hatching eggs.

Key Words: Incubation process, disinfectant, chlorine dioxide, ethanol, cemele pepper.

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INTRODUCTION

During incubation of chicken eggs, newly developing embryos are affected by many factors. These factors either have positive and negative effects on embrionic development. Researhers have developed different strategies to reduce or eliminate factors that negatively affect embrionic development (Ergün *et al*., 2023). Because all of the drawbacks that might occur throughout the incubation period can lead to problems like as developmental delay, embrionic mortalities and reduction in the number of healthy chicks hatched out. Pathogenic microorganisms and abiotic stressors are the primary factors that negatively affect embryo development in incubation period (Ghonım *et al*., 2008; Aygören *et al.,* 2020).

The outer layer of an egg is made of a material with tiny holes. Harmful bacteria on the egg's exterior can enter the egg through these holes when the right temperature and moisture levels are met, leading to infection and death of the growing embryos. (Gole *et al*., 2014). Physical and chemical defence systems protect embryos against pathogen attacks (Chen *et al.,* 2019). However, shell abnormalities and high microorganism density per unit area may result in insufficient physical and chemical defensive mechanisms. Undesirable outcomes such as embryonic mortality and reduced hatchability may arise as a consequence of this circumstance. Therefore, hatching eggs must be

disinfected in order to reduce pathogenic microorganism loads as much as possible before incubation. Disinfection is applied in two ways: dry (fumigation) and wet (spraying or dipping) (Melo *et al.,* 2019; Jabbar *et al.,* 2020). There are many disinfectants that are widely used in poultry industry prior to incubation period. One of the most frequently utilised disinfectants, chlorine dioxide (ClO_2), has a strong oxidative activity, is water-soluble, and is effective against bacteria, yeast, and fungi found on the egg surface (Durmuş, 2012; Hansung *et al.,* 2018).

Conventional disinfection procedures employed in incubation period may produce stress on embryos during developmental stage. This stress is never desirable in order to get the higher quantity and quality of hatching chicks. The primary abiotic stressors during the incubation period include high and low temperatures, insufficient humidity levels, and physical traumas. Stress at any degree may negatively affect embryos and result in poor quality outcomes such as low hatching weight, incomplete umbilical closure, and even deaths. Furthermore, stress-related abnormalities during this period may result in growth retardation, and cardiac and immune system disorders in chicks in their life (Lis *et al.,* 2009).

Many biosafety methods and strategic plans are applied in poultry production to control pathogens throughout the incubation process and alleviate embryonal stress associated with this circumstance (Delpont *et al.,* 2021). However, it is well recognised that some of the chemicals utilised in these methods have carcinogenic and teratogenic effects. This issue has become grounds for a surge in researches into the development of effective natural products with alternative means that will have little or no effect on the disinfection of hatching egg shells, chick embryo development, and health (Batkowska *et al.,* 2018; Oliveira *et al.,* 2020). One of the alternate disinfectants is a pepper plant extract. The pepper (*Capsicum annuum* L.) is a species belonging to the Solanaceae family.Its main component is capsaicin. It is, also, rich in vitamins A, B, C, and E, as well as carotene, polyphenols, flavonoids, minerals, and essential oils (Hernández-Pérez *et al.,* 2020). In addition to its antioxidant capacity it is, also, known to have a bactericidal effect (Lia *et al.,* 2020). Cemele pepper is a bell pepper genotype grown locally in Kırşehir region of Turkey (Ergün, 2021). In this study, it was aimed to investigate the effects of pepper plant extract as an alternative to conventional chemicals used in the disinfection process of hatching chicken eggs on hatching parameters.

MATERIALS AND METHODS

Ethics committee approval no. 06-02 on 31/03/2021 was obtained from the Animal Experiments Local Ethics Committee of Kırşehir Ahi Evran University. The study was carried out July - August 2021 in the laboratory of Animal Physiology and Histology at Kırşehir Ahi Evran University, the Faculty of Agriculture, and the Department of Animal Science under controlled conditions.

Preparation of plant extract: The Pepper (*Capsicum annuum* L.) plant of the Cemele genotype, which is part of the Solanaceae family, was acquired from the local production area in Çayağzı district of Kırşehir province in Türkiye for the study. Peppers at stage of fruit the samples were used slightly red with similar morphological and pigmentation traits were chosen as the plant samples for the research. The samples were initially washed with distilled water and allowed to dry in the shade at room temperature. The drying process was continued until the peppers reached constant weight. The dried samples were physically ground using a grinder. The dried sample (10 grams) was added to a closed Erlenmeyer flask containing 200 mL of 99% methanol (Riedel), and the mixture was stirred with a magnetic stirrer for 3 hours. The mixture was filtered through filter paper. Methanol was removed using an evaporator (40°C). The extract was stored at +4°C until use. The phytochemical properties of pepper extract, the total amount of phenolics equivalent to gallic acid (Sigma) (mg GAE g^{-1}) and total flavonoids equivalent to quercetin (Sigma) (mg QE g^{-1}) were determined (Gulcin *et al*., 2005). DPPH (Sigma) free radical scavenging activities, IC_{50} values and Fe^{3+} and Fe^{2+} reducing power of the samples were determined (Blois, 1958; Oyaizu, 1986). The pepper extract solutions (2% and 4%) used in the study were prepared in ethanol.

Incubation of eggs: 240 hatching eggs which were collected on the same day from Atak-S® layer hen parents (36 wk, body weight 2100-2400 gr) and whose morphological characteristics were observed homogeneously, were used in the study (egg weight 67.03±3.02 g). The eggs were provided from Directorate of Ankara Poultry Research Institute. This study was designed in four groupsT1, T2, T3 and T4, 60 eggs including in each (Ergün *et al.,* 2023). T1 was positive control, sprayed chlorine dioxide solution (40 ml/L water) onto eggs. T2 was negative control sprayed pure ethanol onto eggs. T3 was treatment group (2% pepper extract sprayed onto eggs) and T4 was treatment group (4% pepper extract sprayed onto eggs).

The distribution of eggs among the groups was done at random to ensure homogeneity. Eggs were put in groups on trays, and all groups were disinfected by spraying (Mohammed *et al.,* 2011). Each group received 10 mL of liquid for every 6 eggs. Turoksi brand chloride dioxide (40 mL/L water), one of the chloride group disinfectants, was sprayed as a disinfectant in the positive control group (T1) (Durmuş, 2012), pure ethanol was

sprayed as a disinfectant in the negative control group (T2), and 2% ethanol Cemele pepper extract (T3) and 4% ethanol Cemele pepper extract (T4), which may be used as an alternative to others, were sprayed to the other groups under laboratory conditions. After disinfection, the eggs were left to dry for thirty minutes at room temperature. These eggs were subjected to the incubation for a period of twenty-one days once they were completely dried. During the incubation process, four incubators of the same brand and model were utilised (Cimuka® PD series). The eggs were rotated at an angle of 45 degrees 24 times each day under conditions of a temperature of 37.7°C and a humidity level of 60% for the first 18 days of the acclimatisation process. After that, on the 18th day, the eggs were inspected with light to determine whether or not they were fertile, and then the hatching baskets were subjected to acclimatisation at a temperature between 37 and 37.2°C and the humidity of 70% until all of the eggs had hatched.

Data Collection: This analysis method is based on the principle of determining the number of microorganisms in the environment under aerobic/anaerobic conditions for psychrophile, thermophile or mesophile groups. In the study, a total of four microorganism load counts were made on the 0th, 2nd, 8th and 18th days in order to determine the time-dependent changes of 4 different disinfectants, sprayed to the hatching eggs before incubation, in the microorganism load on the shell. Therefore, three eggs from each group were employed at a time, and in the study carried out at room temperature, each test egg was washed with 10 ml of 0.9% NaCl (normal saline), and the microorganisms on the outer surface of the eggshell were allowed to flow into the solution. Following this treatment, 1 mL (10^0), 10^{-1}, 10^{-2} serial dilutions were prepared in the solution prepared individually for each group, and they were incubated on a commercially manufactured special medium (CompactDry® TC) (Park *et al.,* 2015). After 48 hours of incubation at 37°C, counts were taken, and the values were calculated as cfu/Ml (Özbakır, 2015).

Fertility Rate (FR), Hatchability (H), Discarded Chick Rate (DCR), and hatch of fertile eggs (HFE) were determined in the study (Türker *et al.,* 2018).

Early embryonic mortality (EEM), Middle embryonic mortality (MEM), and "Late embryonic mortality (LEM)" were determined macroscopically at different incubation stages of groups in this study (Birkhead *et al.,* 2008). Consequently, chick quality was determined by utilising hatching weight, chick length, Tona score, and Pasgar score (Boerjan, 2002; Willemsen *et al.,* 2008). Only non-hatching eggs were cracked and examined macroscopically, and malformation and malposition rates associated with death-in-shell were calculated by taking into account the number of fertile eggs incubated in each group (Aşcı and Durmuş, 2015).

The study was conducted to determine the potential stress on the embryos throughout the incubation process, as well as the effect of the plant extracts employed when comparing with the control groups. At the end of the incubation, blood samples were drawn three birds from each group at random, for malondialdehyde (MDA) and reduced glutathione (GSH) analyses in blood serum. The serum was obtained by centrifuging the blood samples at 4 °C for 10 minutes at 1200 rpm, and the serums were stored at +4 °C until the study. (Lotfi *et al*., 2012).

Serum malondialdehyde analysis was done in blood samples drawn from chicks to determine the effectiveness of plant extracts utilised against abiotic and biotic stress factors produced during the incubation. In principle, it is based on the spectrophotometric measurement (Optima SP-3000, Tokyo/Japan) of the pink complex produced by the reaction of malonaldehydes with thiobarbituric acid (İsolab) in a hot and acidic environment. The standard graphic equation constructed by using 1,1,3,3-tetraethoxypropane (Sigma) to calculate serum MDA levels (nmol mL^{-1}) (Jain *et al.,* 1989).

Serum-reduced glutathione assay was done in blood samples drawn from chicks to determine the effectiveness of plant extracts utilised against abiotic and biotic stress factors produced during the incubation process. The Sedlak and Lindsay (1968) method was used to determine the GSH content of serum. The amount of GSH in the serum was determined using the GSH (sigma) standard curve and expressed in μM (Sedlak and Lindsay, 1968).

Statistical Analyses: Data obtained in this study were analyzed by using SPSS 22. V® statistical software package. Samples were collected of microbial load at three different days of incubation, for such data Repeated Measures ANOVA was used. One-Way ANOVA was used for others. (de Souza *et al*., 2017). In circumstances where the differences were determined to be significant, one of the multiple comparison tests, the Duncan's test, was used to identify which treatment or treatments caused this difference (Duggan *et al*., 2017, Genç and Soysal 2018). Differences were ranked at the $P \leq 0.05$ level in the study. The experimental model is as shown below.

$Yij = \mu + \alpha i + eij$

Yij : observed values for traits,
μ : general mean
αi : effect of factors (i=groups; T1, T2, T3, T4)
eij : random residual error

RESULTS

Recently, it has been a matter of great curiosity to investigate the structural potential of plants consumed as food. This situation has increased the studies on plants.

Due to the toxic effects of synthetic antimicrobials, the bactericidal plants has been interested widely.

Antioxidant results of pepper plant: In the current study, Cemele pepper extract was used, instead of a widely employed disinfectant. Total phenolic and flavonoid contents of Cemele pepper extract were determined as 13.44±3.26 µg GAE mL^{-1} and 27.05±16.11 µg QE mL^{-1}, respectively. In addition, IC_{50} value was 394.60±39.81 µg mL-1 and Fe^{3+}-Fe^{2+} reducing power was 69.35±4.25 µg AAE mL^{-1}.

Total mesophilic aerobic bacteria count (TMAB) of the eggshells of test groups were determined on the 0th, 2nd, 8th, and 18th days of the study (Table 1). Before incubation, the TMAB value was found to be 3.45±0.94 log cfu/egg.

Table 1. Total number of mesophilic aerobic bacteria (log cfu/egg) in the eggshell on days 0, 2, 8, and 18.

	Total mesophilic aerobic bacteria (log cfu/egg)			
	Day 0	**Day 2**	**Day 8**	**Day 18**
T1	3.45±0.94	2.60±0.29^{a}	3.66±1.15^{ab}	4.43±0.40^{b}
T2	3.45±0.94	2.54±0.56^{a}	4.33±1.15^{a}	5.36±0.40^{a}
T3	3.45±0.94	1.76±0.42^{b}	2.33±0.57^{bc}	3.26±0.30^{c}
T4	3.45±0.94	0.50±0.14^{c}	1.93±0.20^{c}	2.30±0.20^{d}
P values	-	0.032	0.034	0.028

T1: Control+(chlorine dioxide), T2: Control–(ethanol), T3: ethanol+ 2% Cemele pepper extract, T4: ethanol+ 4% Cemele pepper extract

*: Differences between the means followed by the same letter are not significant at P≤0.05 level

Embryonic mortality of the groups at different stages of incubation is given in Table 2.

Table 2. Embryonic mortality (Early/Mid/Late stage) rates (%) at different incubation stages (days)

	Early stage (1-7 days)	**Mid stage (7-14 days)**	**Late stage (14-20 days)**	**Total embryonic mortality**
T1	4.16±0.00^{b}	10.34±0,30^{a}	12.47±1.17^{a}	25.87±075^{a}
T2	7.11±1.35^{a}	7.11±1.35^{b}	14.22±2.69^{a}	28.55±0.67^{a}
T3	3.70±0.00^{b}	10.91±0.34^{a}	12.66±2.69^{a}	26.31±0,00^{a}
T4	0.00±0.00^{c}	7.11±1.35^{b}	5.30±0.17^{b}	12.66±2.69^{b}
P values	0.001	0.014	0.035	0.024

T1: Control+(chlorine dioxide), T2: Control–(ethanol), T3: ethanol+2% Cemele pepper extract, T4: ethanol+4% Cemele pepper extract

*: Differences between the means followed by the same letter are not significant at P≤0.05

The differences between the groups were determined to be statistically significant at the level of p≤0.05. In the T4 treatment group, no mortality was determined in the early period (days 1-7). The T3 treatment group had the highest mortality of 10.9±10.34% in the middle period (days 7-14). Also, late embryonic mortality (days 14-20) was 14.22±2.69 % in the T2 treatment group, 12.66±2.69 % in the T3 treatment group, 12.47±1.17 % in the T1 treatment group, and 5.30±0.17 % in the T4 treatment group, respectively.

Table 3 shows the fertility rate, hatchability of fertile eggs, and hatchability of totall eggs for all groups. The similarity was determined between the fertility rates of the eggs in the incubation process of the groups, and the differences were statistically insignificant. The differences between the groups in terms of incubation parameters were statistically significant at the level of p≤0.05. The highest hatchability were determined inT4 treatment group.

The differences in live weight (g), body length (cm) and chick quality ratio (Tona Score and Pasgar Score) between the groups were statistically significant at P≤0.05 level. After hatching, the mean chick weights was 38.95±0.85 g in T2 treatment group, 39.44±1.52 g in T3 treatment group, 39.62±1.36 g in T1 group, and 43.57±1.37 g in the T4 treatment group, respectively. Body lengths were determined as the highest value of 16.47±0.45 cm in T4 treatment group and the lowest value as 15.91±1.50 cm in the T3 treatment group. In addition, the highest tona score was 98.10±1.27 at T4 and the lowest tona score was 93.65±1.22 at T1. Pasgar score values of the chicks were 9.69±0.16 at T4, 9.38±0.17 at T2, 9.31±0.18 at T3 and 9.16±0.16 at T1, respectively.

In this study, the number of discarded chicks, as well as the malposition and malformation ratios of disinfection groups were determined (Table 5).

Table 3. Hatching qualities and hatchability table (%) for all treatment groups.

Groups	Macroscopic eggs fertility (%)	Hatchability of fertile eggs (%)	Hatchability of total eggs (%)
T1	95.00±0,00	74.00±3.60^{b}	71.66±1.44^{b}
T2	93.33±2.88	71.30±2.94^{b}	66.66±2.88^{c}
T3	91.66±2.88	72.70±0.84^{b}	66.66±2.88^{c}
T4	93.33±2.88	87.52±2.88^{a}	81.66±1.44^{a}
P values	-	0.011	0.016

T1: Control+(chlorine dioxide), T2: Control–(ethanol), T3: ethanol+2% Cemele pepper extract, T4: ethanol+ 4% Cemele pepper extract

*: Differences between the means followed by the same letter are not significant at P≤0.05

All groups' average body weight (g) and length (cm), as well as their chick quality rates (Tona Score (0-100) and Pasgar Score (0-10) are given in Table 4.

Table 4. Body weight, length and Tona score (0-100) and Pasgar score (0-10) quality values of the hatched chicks.

Groups	Body weight (g) (0d)	Body length (cm) (0d)	Tona score	Pasgar score
T1	39.62±1.36^{b}	16.34±0.57^{a}	93.65±1.22^{b}	9.16±0.16^{b}
T2	38.95±0.85^{b}	16.35±0.52^{a}	95.51±1.36^{ab}	9.38±0.17^{ab}
T3	39.44±1.52^{b}	15.91±1.50^{b}	94.77±1.40^{ab}	9.31±0.18^{ab}
T4	43.57±1.37^{a}	16.47±0.45^{a}	98.10±1.27^{a}	9.69±0.16^{a}
P values	0.011	0.007	0.001	0.008

T1: Control+(chlorine dioxide), T2: Control–(ethanol), T3: ethanol+2% Cemele pepper extract, T4: ethanol+4% Cemele pepper extract

*: Differences between the means followed by the same letter are not significant at P≤0.05

Table 5. Discarded chick, malposition and malformation rates in all treatment groups.

Groups	Discarded chick (%)	Malposition (%)	Malformation (%)
T1	8.59±1.21^{a}	12.01±2.58^{ab}	12.05±1.36^{a}
T2	7.14±0.01^{a}	14.27±0.07^{a}	9.15±3.37^{ab}
T3	5,17±0.15^{b}	10.87±0.31^{ab}	9.24±3.32^{ab}
T4	3.57±0.01^{c}	8.92±0.01^{b}	8.83±0.12^{b}
P values	0.021	0.037	0.018

T1: Control+(chlorine dioxide), T2: Control–(ethanol), T3: ethanol+2% Cemele pepper extract, T4: ethanol+ 4% Cemele pepper extract

*: Differences between the means followed by the same letter are not significant at P≤0.05

The differences between the discarded chick rates of the groups were statistically significant at P≤0.05 level. Discarded chick (%) ratio was 8.59±1.21% in T1 treatment group, 7.14±0.01% in T2 treatment group, 5.17±0.15% in T3 treatment group and 3.57±0.01% in T4 treatment group, respectively. The differences between the groups in terms of malposition and malformation rates were statistically significant at P≤0.05 level. The lowest malposition rate was 8.92±0.01% in T4 and the highest rate was 14.27±0.07% in T4. Malformation ratios were 8.83±0.12% in T4, 9.15±3.37% in T2, 9.24±3.32% in T3 and 12.05±1.36% in T1, respectively.

Chemical Analyses: At the end of the incubation period, blood was drawn from four chicks for biochemical analysis, and the MDA and GSH values of all groups were determined (Table 6).

Table 6. Malondialdehyde (MDA) and reduced glutathione assays (GSH) in blood samples taken from the groups at the end of the incubation period.

Groups	MDA (nmol mL^{-1})	GSH (μM)
T1	8.63±0.05^{a}	13.25 ±1.39^{c}
T2	0.70±0.10^{b}	11.15±1.03^{d}
T3	0.28±0.01^{c}	24.01±1.00^{b}
T4	0.25±0.01^{c}	32.39 ±0.66^{a}
P values	0.018	0.015

T1: Control+(chlorine dioxide), T2: Control–(ethanol), T3: ethanol+2% Cemele pepper extract, T4: ethanol+4% Cemele pepper extract

*: Differences between the means followed by the same letter are not significant at P≤0.05

In the present study, the differences in serum MDA values in newly hatched chicks were statistically significant at the level of P≤0.05. The highest value was 8.63±0.05 nmol mL^{-1} in the T1 treatment group, while the lowest value was 0.25±0.01 nmol mL^{-1} in the T4 treatment group.

GSH results of the treatment groups were statistically significant at the level of P≤0.05. The highest value was 32.39 ±0.66 μM in the T4 treatment group, and the lowest value was 11.15±1.03 μM in the T2 treatment group

DISCUSSION

The current studies has been around on natural products against pathogenic microorganisms due to the toxic effects of antimicrobials. Plants are at the forefront of these resources. Pepper is a powerful antioxidant plant with antimicrobial potential. Previous studies support this approach. A study conducted by Ergün (2021) on Cemele pepper reported that the total amount of phenolic substance was 27.03±0.69 µg GAE mL^{-1}, the total amount of flavonoids was 39.67±2.86 µg QE mL^{-1}, and the IC_{50} value was 195.85 µg mL^{-1}. It was clear that the values determined in the study were lower than the values found in the study conducted by Ergün. These differences may be explained by the planting, production practices , harvesting and storage conditions of the plant (Naznin *et al.,* 2019; Ferysiuk *et al*., 2020).

Before incubation, the TMAB value was found to be 3.45±0.94 log cfu/egg. In similar studies, Harput and Aygün (2021) reported the TMAB value of 4.45 log cfu/egg whereas, Knape *et al*., (1999) reported this value as 3.89 log cfu/egg. The value of 3.45±0.94 log cfu/egg found in the study is lower than the value published by Harput and Aygün (2021) but is partially similar to the value reported by Knape *et al*., (1999). The fact that the determined value is lower than the value reported by Harput and Aygün (2021) is believed to be associated with production, transportation, and storage conditions. On the second day of incubation, significant differences in TMAB values between groups were found ($P \leq 0.05$). The highest TMAB value was recorded in T1 at 2.60±0.29 log cfu/egg, while the lowest value was observed in T4 at 0.50±0.14 log cfu/egg. Measurements taken on the eighth day of ongoing incubation indicated that the differences between groups remained significant at $P \leq 0.05$, with the lowest TMAB value recorded in T4 at 1.93±0.20 log cfu/egg. On the eighteenth day of the incubation process, measurements indicated significant differences between groups ($P \leq 0.05$), with TMAB values measured as follows: T4 at 2.30±0.20, T3 at 3.26±0.30, T1 at 4.43±0.40, and T2 at 5.36±0.40 log cfu/egg, respectively. Low TMAB values are a desired situation in production. Pepper extract was effective in the formation of differences in treatment groups. The results proved that pepper has antibacterial effect. Copur *et al*.(2010) reported that thyme (*Origanum onites L*) essential oil caused the lowest bacterial load for TMAB as 1.36 log cfu/egg. On the other hand, Vilela *et al*.(2012) reported that fungi were more effectively controlled by propolis before incubation than bacteria on eggs.

It has been reported that the application of plant extracts during the incubation process has positive effects on early, middle and late embryo mortality. For example, the use of thyme essential oil for disinfection of chicken eggs caused 3.10% early embryonic mortality and 4.58% late embryonic mortality (Copur *et al*., 2010). Similarly, the use of clove essential oil resulted early mortality rate as 3.03 ± 3.50% and late mortality rate as 4.60 ± 5.95% (Oliveira *et al*., 2020). In the current study, the lowest embryo mortality ratios in all embryonic periods in T4 can be explained by the fact that pepper extract may have a similar effect to thyme essential oil and clove essential oil. In addition, it is thought that the pepper extract may have protected the developing embryos against various stress factors, especially pathogens, which cause mortality during the incubation process. This may be explained by the antibacterial and antioxidant effects of pepper.

It is known that factors such as genetic structure, treatments, care, feeding, storage, environment and incubator are effective on hatchability of eggs (Brake *et al*., 1997; Taylor, 2000; Decuypere *et al*., 2001). Some plant extracts were used by dipping method to get the higher hatchability of fertilised eggs as 97.26% (fenugreek, *Trigonella foenum-graecum* L.), 94.44% (oat, *Avena sativa* L.) and 94.50% (basil, *Ocimum basilicum*) (Al-Asadi and Ibrahim, 2020). Similarly, the hatching efficiency of total incubated eggs was determined as 94.73% (fenugreek, *Trigonella foenum-graecum* L.), 92.53% (oat, *Avena sativa* L.) and 93.20% (basil, *Ocimum basilicum*) (Al-Asadi and Ibrahim, 2020). N'nanle *et al*. (2017) reported that the hatchability of fertile eggs in the group treated with 0.5 µg mL-1 *Moringa oleifera* was 93.8%. In our study, however, the highest hatching quality was found in T4 treatment group as 87.52 %. This lower value compared to those of previous studies can be explained by the different treatments and plant extracts applied to the eggs.

In the study, the highest mean weight and body length were determined in T4 chicks. This may be explained by the fact that the pepper extract may have shown nutritive affects on embryo when propolis disinfected hatching eggs, the average live weight of the chicks was found as 44.04 g (Mousa-Balabel *et al.*, 2016). Also, the ethanolic propolis extract and ginger oil increased live weight of newly hatched chicks (Debes and Basyony, 2011; Shahein and Sedeek, 2014; Oliveira *et al*., 2020).

Various methods have been used to determine chick quality. In this study, Tona and Pasgar scores were used to determine chick quality. A Tona score close to 100 and Pasgar score close to 10 means high quality. In addition, a Tona score between 80 and 100 and Pasgar score between 8 and 10 are considered to be high quality chicks (Boerjan, 2002; Willemsen *et al*., 2008). In this study, the highest Tona Score value (98.10±1.27) was found in T4 group and the highest Pasgar Score value (9.69±0.16) was found in T4 group. Tona *et al*.(2005) reported that in ovo feeding and optimal hatching conditions both increase embryonal protein synthesis and consequently affect chick quality positively.

Considering the discarded chick rates, T4 treatment group was the lowest with 3.57±0.01%. The lowest malposition rate 8.92±0.01, and the lowest malformation rate 8.83±0.12% were observed in T4 treatment group. Similarly, thyme (*Origanum onites* L.) oil lowered discarded chick rate (Copur *et al*., 2010). In a different study on quail (*Coturnix coturnix japonica*) eggs, garlic (*Allium sativum* L.) extracts had no effect (Baylan *et al.,* 2018).

MDA level in serum is an indicator of tissue damage. MDA is, also, one of the products of lipid peroxidation. If this value is high, it means that the damage in the tissues is high. The serum MDA value of newly hatched chicks was reported as 0.59±0.06 nmol/ml by Altan *et al*. (2017) and 10.73 nmol/ml by Lotfi *et al*. (2012). Also, serum MDA value was reported as 4.05 - 3.45 nmol/ml in a different study by El-Fakhrany *et al*. (2021). In the current study, the lowest MDA level was found as 0.25 ± 0.01 nmol/ml in the T4 treatment group. This decrease may be due to the antioxidant content of pepper extract. This shows that the oxidative damage caused by lipid peroxidation might have been prevented.

Increased stress factors enhance reactive oxygen species (ROS) in the environment. This situation causes embryos to be exposed to oxidative stress during the incubation process and damage to their development (Suari *et al*., 2018). Developing embryos need to synthesise GSH to overcome this situation (Métayer *et al*., 2008; Bunchasak, 2009). Glutathione (GSH) is one of the main metabolites formed in embryos during incubation. GSH concentration determine the antioxidant status in the body (Bin *et al*., 2017). The presence of antioxidants in the environment causes an increase in GSH level. In this study, the amount of GSH was determined as 32.39±0.66 µM in T4, 24.01±1.00 µM in T3, 13.25±1.39 µM in T1 and 11.15±1.03 µM in T2, respectively. Also, GSH level increased with the increased extract concentration. This might be explained that the antioxidant effect of the plant extract mihgt have penetrated through the pores in the egg shell.

Conclusion: In this study, the effects of cemele pepper extract on the hatchability of chicken eggs were investigated. There was no undesirable results due to the use of pepper extract. Results showed that 4% pepper extract spraying onto eggs reduced the total eggshell bacterial load during hatching process. In addition, pepper extract application increased hatching efficiency, hatchability and chick quality while it decreased the number of discarded chick, early, middle and late mortality. To conclude, the use of Cemele pepper extract at 4% during the incubation process of chicken eggs had positive outcomes, having a potetial to affect poultry sector whereas more studies are needed for the widespread industrial use.

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