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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

The Effects of Black Mulberry (*Morus nigra* L.) Leaf Powder on Performance, Egg Quality, Egg Antioxidant Capacity, and Yolk Cholesterol Levels in Laying Hens

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Abstract: Different antioxidant substances are supplemented to prevent oxidative stress and improve egg quality in poultry nutrition. It is known that synthetic antioxidants negatively affect human health. Therefore, natural antioxidants are often investigated. In this study, the possibilities of using different levels of black mulberry (*Morus nigra* L.) leaf powder (BML) in laying hens were examined. For this purpose, 3 groups were formed, 24 in each group, and a total of 72 hens (35 weeks-old, Brown Nick) were distributed to individual cages. The control group (BML-0) was fed using a basal diet. Experimental groups were fed using 0.3 and 0.6 g/kg BML supplementation to the basal diet, respectively. Performance, egg quality characteristics, yolk TBARS (thiobarbituric acid reactive substances), DPPH (2,2-diphenyl-1-picrylhydrazyl), and cholesterol levels were evaluated in the trial, which lasted for a total of 9 weeks, one week of which was the acclimatization period. There was no statistically significant difference between the groups in terms of performance, egg quality characteristics, and DPPH reducing % in yolk (P>0.05). Yolk TBARS and cholesterol levels were significantly affected from supplemented-BML diet (P<0.05). It was determined that the cholesterol level of yolk decreased linearly with the addition of BML (P<0.05). It was determined that the BML addition delayed lipid oxidation due to decreased TBARS value in the yolk. As a result, it was determined that black mulberry leaves can be used to obtain low-cholesterol eggs and delay lipid oxidation without negatively affecting performance parameters in laying hens. It is thought that it will be useful to reveal the effects of black mulberry leaf on different parameters in future studies.

Keywords: Antioxidant, black mulberry, cholesterol, egg quality, TBARS.

Karadut (*Morus nigra* L.) Yaprak Tozunun Yumurtacı Tavuklarda Performans, Yumurta Kalitesi, Yumurta Antioksidan Kapasitesi ve Kolesterol Düzeyine Etkileri

Öz: Oksidatif stresi önlemek ve yumurta kalitesini geliştirmek için kanatlı karma yemlerine farklı antioksidan maddeler ilave edilmektedir. Sentetik antioksidanların insan sağlığını olumsuz olarak etkilediği bilinmektedir. Bu nedenle doğal antioksidanlar sıklıkla araştırılmaktadır. Bu çalışmada karadut (*Morus nigra* L.) yaprak tozunun (BML) farklı düzeylerinin yumurtacı tavuklarda kullanım olanakları ortaya konulmuştur. Bu amaçla her bir grupta 24'er adet olmak üzere 3 grup oluşturulmuş ve toplam 72 adet tavuk (35 haftalık, Brown Nick) bireysel kafeslere dağıtılmıştır. Kontrol grubu (BML-0), bazal yem ile beslenmiştir. Deneme grupları ise bazal yeme sırasıyla 0.3 ve 0.6 g/kg BML ilavesi ile beslenmiştir. Bir haftası alıştırma periyodu olacak şekilde toplam 9 hafta süren denemede performans, yumurta kalite özellikleri, yumurta sarısı TBARS (tiyobarbitürik asit reaktif maddeler), DPPH (2,2-diphenyl-1-picrylhydrazyl) ve kolesterol düzeyleri değerlendirilmiştir. Performans, yumurta kalite özellikleri ve yumurta sarısında DPPH % indirgeme gücü bakımından gruplar arasında istatistiksel olarak anlamlı bir fark olmadığı tespit edilmiştir (P>0.05). Yumurta sarısı TBARS ve kolesterol düzeyleri ise karma yemdeki BML'den önemli düzeyde etkilenmiştir (P<0.05). BML ilavesi ile yumurta sarısı kolesterol düzeyinin linear olarak azaldığı tespit edilmiştir (P<0.05). BML ilavesinin yumurta sarısında TBARS değerinin azalması sonucunda lipid oksidasyonunu geciktirdiği belirlenmiştir. Sonuç olarak, yumurtacı tavuk karma yemlerindeki karadut yaprağının performans parametrelerini olumsuz yönde etkilemeden düşük kolesterollü yumurta eldesinde ve lipid oksidasyonunun geciktirilmesinde kullanılabileceği görülmektedir. İleriki çalışmalarda karadut yaprağının farklı parametreler üzerindeki etkilerinin ortaya konulmasının faydalı olacağı düşünülmektedir.

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Anahtar kelimeler: Antioksidan, karadut, kolesterol, yumurta kalitesi, TBARS.

INTRODUCTION

Various feed additives are used to increase the quality of animal products, improve performance, and protect animal health in poultry (Pirgozliev et al., 2019). The use of synthetic antioxidants as feed additive is controversial due to their potential negative effects (Lorenzo et al., 2018). The use of antibiotics to promote growth has been banned since 2006 (Tian et al., 2021). For these reasons, researchers and manufacturers have returned to natural additives (Placha et al., 2022). One of these natural additives that have plant origins (Yitrabek, 2015) is called phytobiotics or phytogenic additives (Bagno et al., 2018). Plants have different properties such as antioxidant, antimicrobial, antiviral, anti-inflammatory, anticancer, and antidiabetic thanks to the secondary metabolites they contain (Jain et al., 2019). There are different studies carried out on the use of several phytobiotics such as jujube leaf (Kılınç et al., 2020), thyme (Ghasemi et al., 2010; Mansoub, 2011; Mohammed et al., 2022), sage (Assi-Husain et al., 2023), black cumin (Ghosh et al., 2020; Ali et al., 2023), nettle (Parizadian Kavan et al., 2023), garlic (Ghasemi et al., 2010; Ghosh et al., 2020), rosemary (Çufadar, 2018; Cimrin, 2019), ashwagandha (Sandeep et al., 2020; Kılınç, 2023), anise (Yu et al., 2018), and wild leek (Kılınç et al., 2023) in laying hens. One of the subjects investigated as a natural additive is mulberry. Mulberry (*Morus* spp.) belong to in the Moraceae family (Zhang et al., 2018). This genus has many species (Sánchez, 2000; Yigit et al., 2010). It is known that white mulberry (*Morus alba* L.), black mulberry (*Morus nigra* L.), and red mulberry (*Morus rubra* L.) are common (Butkhup et al., 2013). The high antioxidant content of black mulberry (*Morus nigra* L.) leaves was reported in previous studies (Radojković et al., 2018; Polumackanycz et al., 2021; Vukmirović et al., 2023). There are many studies investigating *Morus alba* L. leaves in laying hens (Al-kirshi et al., 2010; Olteanu et al., 2012; Kamruzzaman et al., 2014; Lin et al., 2017; Zhang et al., 2022). However, as can be seen in the literature, the number of studies investigating the possibilities of using *Morus nigra* L. leaves in poultry is limited. Therefore, the present study examines the effects of black mulberry leaf powder (BML) on performance, egg quality characteristics, yolk TBARS level, DPPH radical scavenging activity (% reduction), and cholesterol level in laying hens.

MATERIAL AND METHOD

Preparation of Black Mulberry Leaf Powder: Black mulberry leaves collected in June were dried under the shade conditions. The dried leaves were ground in a laboratory blender and stored under suitable conditions until the experiment.

Experiment Plan and Feeding of Animals: The animal experiment was carried out at Amasya University Suluova Vocational School for 9 weeks, one week of which was an acclimatization period. Brown Nick hens (35- weeks old) were divided into 3 groups (total 72 hens), 24 in each group, and distributed in 4-storey cages (35 cm × 40 cm × 40 cm) (Table 1). Hens were put into a completely randomized block design. The number of hens in groups was determined using G*Power 3.1. program's F tests (Fixed effects, omnibus, one-way) module. While the chickens were placed in the cages, it was tested that the groups were homogeneous in terms of weight (P>0.05). During the experiment, feed and water were given *ad libitum*. Lighting was performed for 16 hours daily (lights on at 5:30 and lights off at 21:30). The temperature and humidity levels were routinely checked during the experiment.

Table 1. Experiment plan

Groups	Number of Repetitions (n)	Feed Additive
BML-0	24	-
BML-1	24	0.3 g/kg BML
BML-2	24	0.6 g/kg BML

BML: Black Mulberry Leaf.

The control group (BML-0) was fed a basal diet. Experimental groups (BML-1 and BML-2) were fed individually by adding 0.3 and 0.6 g/kg black mulberry leaf (BML) powder to the basal diet, respectively. The chemical composition of the basal diet and BML powder were determined by the method reported in AOAC (2000). The components and chemical composition of the basal feed used in the experiment are presented in Table 2. The nutrient composition of BML powder analyzed is given in Table 3.

Table 2. Chemical composition of basal diet used in the experiment

Ingredients	kg/ton	Nutrient contents (analyzed)	%
Maize	500.000	Dry matter	88.96
Soybean meal (46% CP)	138.130	Crude protein	17.50
Full-fat soybean (34% CP)	94.111	Crude fibre	4.19
Sunflower meal (34% CP)	86.471	Ether extract	4.35
Triticale	73.380	Crude ash	12.24
Sunflower oil	6.744	Methionine	0.37
Limestone	87.265	Lysine	0.85
Dicalcium phosphate	8.242	Calcium	3.6
Salt	3.246	Methionine + Cystine	0.69
DL-methionine	0.011	Tryptophan	0.22
Premix*	2.500	Arginine	1.22
Total	1000	Threonine	0.69
Calculated ME (kcal/kg)**		2760	

*Each 2.5 kg of premix contains 10.000.000 IU Vitamin A, 3.000.000 IU Vitamin D3, 25.000 mg Vitamin E, 3.000 mg Vitamin K3, 2.000 mg Vitamin B1, 5.000 mg Vitamin B2, 40.000 mg Vitamin B3, 12.000 mg Vitamin B5, 4.000 mg Vitamin B6, 1.000 mg Vitamin B9, 60 mg Vitamin H, 20 mg B12, 120.000 mg Mn, 40.000 mg Fe, 70.000 mg Zn, 7.000 mg Cu, 1.000 mg I, 500 mg Se, 2.500 mg canthaxanthin, apo-carotenoic acid ester. CP: Crude protein.
**Calculated metabolic energy (TSI, 1991).

Table 3. Chemical composition of BML powder

Nutrient contents (analyzed)	%
Dry matter	93.7
Crude protein	15.5
Ether extract	1.5
Crude fibre	7.0
Crude ash	16.2

Determination of Performance Parameters: Body weight gain (g), egg weight (g), feed consumption (g), egg production (%), and feed conversion ratio (g feed/g egg) were determined as performance parameters. The remaining feed and egg weights were measured every 2 weeks. Performance parameters were determined using all these data. After subtracting the remaining feed amount from the total amount of feed given, it was divided by the number of days and the daily feed consumption of each group was calculated. Egg production was calculated by dividing the total egg number of each group by the number of animals in that group and multiplying by 100. The feed conversion ratio was determined by dividing the feed consumed for 2 weeks by egg weight. The body weight gains of the hens weighed at the beginning and the end of the trial were calculated (Body weight gain = Final body weight – Initial Body weight).

Determination of Egg Quality Parameters: In the present study shape index (%), yolk index (%), albumen index (%), Haugh unit (%), yolk color (L*, a*, and b* values), shell weight (g), and shell thickness (µm) were evaluated as egg quality characteristics. For this purpose, eggs collected in the last two days of the experiment (n=40) were used. Egg and eggshell weights were determined with a precision scale of 0.001 g. Egg width, length, yolk diameter, and albumen width and length were measured with digital caliper (1108-150, Insize, China). Yolk height and albumen height were measured using a tripod micrometer (Mitutoyo, 0.01–20 mm; Kawasaki, Japan). Shape index (Duman et al., 2016), yolk index (Şekeroğlu & Altuntaş, 2009), albumen index (Iskender et al., 2017), and Haugh unit (Ryu et al., 2011) were calculated using the formulas given below.

Shape index (%) = [egg width/egg length] × 100

Albumen index (%) = [albumen height] / [(albumen length + albumen width) / 2] × 100

Yolk index (%) = [yolk height/yolk width] × 100

Haugh unit (%) = 100 log (H + 7.57 - 1.7W$^{0.37}$), (W = egg weight; H = albumen height)

Eggshell thickness was measured using a micrometer (Mitutoya, 0.01-5 mm; Kawasaki, Japan) in three different parts (upper and lower ends and middle). The colorimeter (PCE-CSM 4) was used to determine yolk color values (L*, a*, and b*). L* value indicates lightness, a* value indicates redness, and b* value indicates yellowness (Lokaewmanee et al., 2013).

Determination of Yolk TBARS Value: As an indicator of lipid oxidation, the MDA (malondialdehyde) value was determined in 18 eggs (n=18) from each group at the end of the experiment (Kılıç & Richards, 2003). In this method, 2g yolk is taken and added with 12 ml TCA solution (7.5% TCA, 0.1% EDTA, 0.1% propyl gallate) and homogenized for 20-25 sec. in ultra-turrax. Then, they were filtered using the Whatman 1 filter paper. Then, 3 ml of filtrate was taken and put into glass tubes and added with 3 ml 0.02 M TBA solution. These tubes containing solution were kept in a water bath at 100°C for 40 minutes and then cooled under tap water. Cooled tubes were centrifuged at 2000 rpm for 5 minutes and the absorbance values were measured at 530nm wavelength using a spectrophotometer (Genesys 10S UV-VIS, Therma Scientific, USA). TBARS value (µmol MDA/kg egg) was calculated with the help of the formula below.

TBARS=[(absorbance/k(0.06)×2/1000)×6.8]×1000/sample weight

k= Value obtained from the standard curve

(TBARS: Thiobarbituric acid reactive substances)

Determination of Yolk DPPH Value: The method of Farivar (2014) was used to determine DPPH (2,2-diphenyl-1-picrylhydrazyl) the radical scavenging activity (DPPH reduction, %) in 18 yolks (n=18) from each group at the end of the experiment. In this method, 2g yolk was added with 25 mL methanol in falcon tubes and the extraction process was performed for 20 minutes in an ultrasonic bath (Elmasonic S 80/H). Yolk-methanol mixture was filtered using filter paper. Filtrates were pipetted to 0.1 ml glass tubes. Then, they were added with 2.9 mL DPPH solution (100 mL Methanol + 0.0025 g DPPH). Tubes were mixed for 25 sec. in a vortex (IKA Vortex 4 Basic), kept under dark conditions for 1 hour, and then sample absorbances and control tubes were read using a spectrophotometer (Genesys 10S UV-VIS, Therma Scientific, USA) at 517 nm wavelength. DPPH value was calculated with the help of the following formula (Sudha et al., 2011).

DPPH (%) = [(A0-A1)/A0] × 100

(A0: Absorbance of the control, A1: Absorbance of the sample)

Determination of Yolk Cholesterol Value: Yolk cholesterol level was determined using 10 eggs (n=10) from each group at the end of the experiment. Yolk cholesterol was determined by the colorimetric method (Washburn & Nix, 1974).

Statistical Analysis: Analysis of variance (One-Way Analysis of Variance), intergroup comparisons between (Duncan's test), and polynomial analysis (linear and quadratic effects) were performed using the SPSS 21.0 package program. The effects (significance) of the groups were evaluated at $P<0.05$ level (IBM Corp., 2012). In addition, the number of hens used in the present study was determined by the G*Power (Faul et al., 2007) software package (version 3.1.4).

RESULTS AND DISCUSSION

Performance Parameters: The results regarding the performance parameters [Initial Body Weight (IBW), Final Body Weight (FBW), Body Weight Gain (BWG), Egg Weight (EW), Egg Production (EP), Feed Intake (FI), and Feed Conversion Ratio (FCR)] are presented in Table 4.

The effects of BML powder (0.3 and 0.6 g/kg) on performance parameters were found to not be statistically significant in laying hens (P>0.05). The results achieved here are consistent with previous studies reporting that mulberry leaves do not affect performance parameters in laying hens (Olteanu et al., 2012; Panja, 2013; Kamruzzaman et al., 2014).

Egg Quality Parameters: The results achieved here regarding egg quality traits [Shape Index (SI), Yolk Index (YI), Albumen Index (AI), Haugh Unit (HU), Shell Weight (SW), Shell Thickness (ST), Lightness (L*), redness (a*), yellowness (b*)] are given in Table 5.

The effects of BML powder supplemented to the diet of laying hens on egg quality parameters were statistically insignificant (P>0.05). In the polynomial analysis, the linear effect was found significant in terms of YI, HU, and a* values (P<0.05). These values have linearly increased with BML levels. In a previous study (Kamruzzaman et al., 2014), it was determined that mulberry leaves (3, 6, and 9%) did not affect egg quality characteristics. In another study, it was reported that ST, HU, and SW were not affected by mulberry leaves (3 and 6%) in their diet (Olteanu et al., 2012). These findings are consistent with the results of the present study. Contrary to the present study, it was determined that mulberry leaves increased ST (Lin et al., 2017; Huang et al., 2022), HU (Lin et al., 2017), yolk color (Olteanu et al., 2012; Xue-dong et al., 2013; Lin et al., 2017), and the SW (Lin et al., 2017).

Yolk TBARS Value, DPPH Radical Scavenging Activity, and Cholesterol Value: The results of the study on yolk TBARS, DPPH, and cholesterol values are given in Table 6.

Table 4. Effect of BML powder on performance parameters (n=24)

Parameters	Groups			SEM	P values		
	BML-0	BML-1	BML-2		C	L	Q
IBW	1772.61	1794.21	1768.58	17.034	0.808	0.924	0.520
FBW	1791.26	1844.50	1816.88	18.074	0.492	0.566	0.297
BWG	18.65	50.29	48.29	8.146	0.209	0.139	0.330
EW	61.49	61.31	61.74	0.203	0.687	0.619	0.478
EP	89.41	86.71	86.35	0.818	0.238	0.122	0.491
FI	117.31	117.14	114.00	0.756	0.132	0.074	0.354
FCR	1.91	1.92	1.85	0.013	0.083	0.063	0.217

BML-0, BML-1, BML-2: Groups fed black mulberry powder added to basal diet at 0, 0.3, and 0.6 g/kg levels, respectively. SEM: Standard Error of Mean; C: Combined; L: Linear; Q: Quadratic; IBW: Initial Body Weight, g; FBW: Final Body Weight, g; BWG: Body Weight Gain, g; EW: Egg Weight, g; EP; Egg Production, %; FI: Feed Intake, g/day/hen; FCR: Feed Conversion Ratio, g feed/g egg.

Table 5. Effects of BML powder on egg quality characteristics (n=40)

Parameters	Groups			SEM	P values		
	BML-0	BML-1	BML-2		C	L	Q
SI	79.32	78.80	79.53	0.233	0.421	0.724	0.206
YI	44.30	44.92	45.31	0.205	0.127	**0.044**	0.777
AI	9.16	9.52	9.59	0.120	0.295	0.148	0.557
HU	83.85	85.83	86.06	0.451	0.088	**0.045**	0.867
SW	7.05	7.09	7.03	0.048	0.877	0.849	0.634
ST	0.364	0.369	0.360	0.002	0.245	0.388	0.151
L*	54.40	53.63	53.66	0.210	0.240	0.251	0.374
a*	11.36	11.72	11.83	0.091	0.092	**0.037**	0.505
b*	43.84	43.49	44.10	0.227	0.557	0.646	0.328

BML-0, BML-1, BML-2: Groups fed black mulberry powder added to basal diet at 0, 0.3, and 0.6 g/kg levels, respectively. SEM: Standard Error of Mean; C: Combined; L: Linear; Q: Quadratic; SI: Shape Index, %; YI: Yolk Index, %; AI: Albumen Index, %; HU: Haugh Unit, %; SW: Shell Weight, g; ST: Shell Thickness, µm; L*: Lightness; a*: Redness; b*: Yellowness.

Table 6. Effect of BML powder on yolk TBARS (n=18) and DPPH (n=18), cholesterol (n=10) values

Parameters	Groups			SEM	P values		
	BML-0	BML-1	BML-2		C	L	Q
TBARS (µmol MDA/kg egg)	3.009[a]	1.768[b]	1.865[b]	0.182	**0.006**	**0.027**	0.063
DPPH (%)	7.928	7.937	8.555	0.474	0.832	0.599	0.768
Cholesterol (mg/g)	12.70[a]	11.45[b]	11.16[b]	2.407	**0.015**	**0.007**	0.299

a,b: The averages with different superscripts in the same row differ significantly (P<0.05).
BML-0, BML-1, BML-2: Groups fed BML powder added to basal diet at 0, 0.3, and 0.6 g/kg levels, respectively. 0, 0.3, and 0.6 g/kg levels, respectively. SEM: Standard Error of Mean; C: Combined; L: Linear; Q: Quadratic; TBARS: Thiobarbituric acid reactive substances; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

Although different levels of BML powder did not affect DPPH radical scavenging activity in yolk, it affected TBARS and cholesterol levels (P<0.05). It was determined that adding BML to the diet had a positive effect by reducing the yolk TBARS value and cholesterol level. In the polynomial analysis, linear and quadratic effects were significant found to be in terms of yolk TBARS and cholesterol (P<0.05). The decrease in TBARS in yolk is thought to originate from the antioxidant property of BML. The previous studies emphasized that the antioxidant properties of BML are high (Polumackanycz et al., 2021; Vukmirović et al., 2023). Johri et al. (2013), in their study, associated the decrease in the cholesterol level of the yolk with the hypocholesterolemic effect of the mulberry leaf. Therefore, it is thought that the decrease in the cholesterol level of the yolk in the present study is because of the hypocholesterolemic effect of the black mulberry leaf. Lin et al. (2017) reported that mulberry leaf extract at different levels (0.5, 1, and 2%) decreased the serum MDA level. In another study, Zhang et al. (2022) determined that different mulberry leaf extract levels (0.4, 0.8, and 1.2%) reduced the cholesterol levels of eggs. Furthermore, Kamruzzaman et al. (2014) reported that different levels (3, 6, and 9%) of mulberry leaf meal reduced the cholesterol level in the yolk. The results of the present study overlap with these studies.

CONCLUSION

BML powder at different levels (0.3 and 0.6 g/kg) in diets of laying hens did not significantly affect other parameters (performance, egg quality traits, and yolk DPPH % reduction), except TBARS and cholesterol level in yolk. It was determined that BML delayed lipid oxidation by decreasing MDA in the yolk. In addition, it was also determined that the BML powder reduced the cholesterol level in eggs compared to the control group. Given the findings of the present study, it was concluded that BML can be used to prevent lipid oxidation and obtain low-cholesterol eggs without negatively affecting performance parameters. However, it is thought that more comprehensive studies on BML would be beneficial.

ETHICS COMMITTEE PERMISSION

The Ethics Committee approval for this study was obtained from Ondokuz Mayıs University Animal Experiments Local Ethics Committee (2023/10).

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