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Effects of Dietary Supplementation of Milk Thistle and Nettle Essential Oils on Performance, Egg Quality, and Hematological Parameters in Layer Hens

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Abstract
This study was conducted to evaluate the effects of dietary levels of milk thistle (MTEO), or nettle essential oils (NEO) on production performance, egg quality and some serum metabolites of white layer hens (Hy-line W-36) aged 60 weeks. A total of 90 birds were randomly assigned to five groups with six replicates of three hens each to study the impact of five dietary treatments consisting of control (as basal diet) and dietary supplementation of different levels of MTEO and NEO (100 and 200 mg/kg). During the experiment period, birds had free accece to feed and water. Dietary supplementation of NEO and 200 mg/kg of MTEO improved egg weight compared to birds that received 100 mg/kg of MTEO ($P < 0.05$). Also, egg production was greater in 200 mg/kg MTEO treatment than those of birds under control and 100 mg/kg of NEO. Adding MTEO and NEO to the diet exhibited no significant influence on egg mass, feed conversion ratio , and feed intake in laying hens. No change in egg quality characteristics and serum parameters was found in birds fed with MTEO- and NEO-supplemented diets. Diets supplemented with 200 mg/kg of MTEO decreased the percentage of heterophils and heterophil to lymphocyte ratio when compared to control and 200 mg/kg of NEO ($P < 0.05$). In conclusion, dosage of 200 mg/kg of MTEO could be considered as an dietary feed additive to improve the egg production and immune response in laying hens.

Introduction
In the last decades, many countries have tended to minimize or ban chemical components and antibiotics in poultry nutrition for their deleterious side effects on the health of both animals and consumers (Brenes and Roura, 2010). Meanwhile, considerable research has been focused on the possible utilization of natural agents, such as oils and extracts of medicinal plants, and spices (Kralik *et al*., 2015). For a long time, medicinal herbs have been traditionally used in the treatment of some diseases in the world (Wallace *et al*., 2010). The mode of function for most medicinal plants is still not entirely recognized. However, the immunomodulatory, antioxidative, growth-stimulating and antimicrobial influences have been presented by several investigators in animals. (Abdel-Hack and Alagawany, 2015; Boka *et al*., 2014; Zhao *et al*., 2013). Essential oils (EO) are usually the main constituent of the medicinal, accounting for most of the positive effects of these herbs (Dorman *et al*., 2000). During the last decade, the EO of certain medicinal plants gets high in regard as a possible growth supplement in poultry generation, particularly for broilers (Boka *et al*., 2014; Zhao *et al*., 2013). Nevertheless, the research results on dietary effects of EO in laying hens are scarce. Some hopeful findings were reported by Bolukbasi *et al*., (2008, 2009) in immune response, yield, and eggshell quality of laying hens fed diets additive with essential oil of sage, thyme, and rosemary.

Nettle (*Urtica dioica* L.), a medicinal herb belonging to the *Urticaceae* family, is a perennial plant growing in different parts of the world, in particular, in temperate and tropical wastelands

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(Chaurasia and Wichtl, 1987). This plant is traditionally used for its anti-oxidative and growth-stimulating properties (Toldy *et al*., 2005). Nettle contains compounds such as starch, gum, albumen, sugar, resin, histamine, acetylcholine, choline, and serotonin (Loetscher *et al*., 2013). Hosseini mansoub (2011) stated that dietary inclusion of nettle in laying hens makes better performance and decreases serum total triglycerides, cholesterol, and low-density lipoprotein (LDL) concentration.

Milk thistle (Silybum marianum) is an herbaceous perennial plant, a member of the Carduusmarianum family, which is used in the therapy of liver disease (Qavami *et al*., 2013). The primary active components of milk thistle are flavonolignans, collectively known as silymarin which is known as a mixture of silybin, silydianin, and silycristin isomers. The medicinal properties of this plant are mainly because of the existence of silymarin. The seeds contain the highest amount of silymarin (Qavami *et al*., 2013). Hashemi Jabali *et al*., 2018 found thatlaying hens fed a ration supplemented with milk thistle meal showed improved feed conversion ratio compared to the hens fed free milk thistle meal diet. Previous literature showed that a diet added with Silybum marianum extract increased daily weight and improved feed conversion ratio (FCR) in broiler chickens (Zarei *et al*. 2016). Despite the promising effects of MTEO and NEO in a few studies with laying hens, their influence on the same birds needs to be characterized in detail. Therefore, in the present research, the effect of dietary levels of MTEO and NEO on performance, blood constituents, and egg quality in laying hens was investigated.

Materials and Methods

Birds and housing

A total of ninety 58-week-old white laying hens (Hy-line W-36) were distributed into 30 wire cages and reared for 8 weeks (including an adaptation period of 2 weeks). All birds were weighed individually at the initiation as well as at the end of the experiment. During the experimentation period, temperature and relative humidity were maintained at approximately 22°C and 55%, respectively. The adjustable lighting was 16 h of continual light from 6:00 A.M to 10:00 P.M daily. Each cage was provided with a nipple drinker and a metal chicken feeder. The cage size was 30.5 × 40.6 cm, providing 413 cm^2 of space per bird.

Experimental diets

Birds were fed with a corn-soybean meal diet *ad libitum*. Diets were formulated according to the instruction in the commercial administration guide (Hy-Line International, Des Moines, IA). The chemical composition and components of the basal diets are revealed in Table 1. Five treatments consisted of a basal diet in mash physical form without and with dietary levels of MTEO and NEO (100 and 200 mg/kg). EOs were added to soybean oil and then blended with corn. Finally, all ingredients were blended.

Table 1. Ingredients and main nutrient composition of basal diet

Ingredients	Amount (%)
Corn	60.00
Soybean meal	25.00
Wheat bran	1.40
Soybean oil	2.92
Dicalcium phosphate	0.9
Limestone	9.00
Salt	0.28
Vitamin premix	0.25
Mineral premix	0.25
Calculated composition	
Metabolizable energy (Kcal/kg)	2842.66
Crude protein (%)	16.33
Lysine (%)	0.82
Methionine+ cystine (%)	0.52
Calcium (%)	3.68
Available phosphorus (%)	0.30

Each kg of vitamin premix contained: Vitamin A, 8,500,000 IU; Vitamin D_3, 2,000,000 IU; Vitamin E, 9000 IU; Vitamin K_3, 2200 mg; Vitamin B_1, 1000 mg; Vitamin B_2, 3,000 mg; Vitamin B_3, 5,000 mg; Vitamin B_5, 25,000 mg; Vitamin B_6, 150 mg; Vitamin B_9, 500 mg; Vitamin B_{12}, 7.5 mg; Biotin, 500 mg; Choline chloride, 250,000 mg. Each kg of mineral premix contained: Mn, 50,000 mg; Fe, 25,000 mg; Zn, 50,000 mg; Cu, 5,000 mg; I, 500 mg; Se, 100 mg.

Performance and egg production

Feed intake (FI) was measured weekly. FCR was estimated as g of feed intake per g of egg production. Eggs were gathered daily, and egg production was calculated on a bird-day grade. Each replicate's egg mass was determined by multiplying egg production by mean egg weight.

Hen-Day-Egg Production

$$(\text{HDEP}) = \frac{\text{Total number of eggs produced on a day}}{\text{Total number of hens present on that day}} \times 100$$

Egg quality

Twelve eggs from each treatment were randomly collected twice a week to evaluate the egg quality index, including shell thickness and strength, Haugh unit (HU), and yolk color. eggs were individually weighed and broken. Eggshell strength was estimated by an eggshell strength tester (Ogawa Seiki Co., Tokyo, Japan). At three points (top, middle, and bottom) eggshell thickness was estimated with an electronic micrometer (Ogawa Seiki Co., Tokyo, Japan). Using a Roche color fan, yolk color was evaluated. HU was estimated from the data of albumen height and egg weight via following formula; Haugh Unit= 100 log10 (H - 1.7 W 0.37 + 7.56), where, W = egg weight (g), and H = height of the albumen (mm).

Blood components

At the end of the investigational period, blood sample was collected from the wing veins of 10 birds per treatment. Samples were then placed in two test tubes with and without ethylenediaminetetra acetate (EDTA) as an anticoagulant. Blood samples were centrifuged for 10 min at 2,000 × g and the collected sera were stored at -20 ºC pending biochemical analysis. An automatic blood analyzer (Boehringer Mannheim, Ingelheim am Rhein, Germany) was applied to estimate blood serum concentrations of cholesterol, glucose, triglyceride, total protein, and albumin. Tubules containing anticoagulants were used to differentiate white blood cells. Blood smears were provided on slides and colored by Giemsa procedure. Using an optical microscope at least 100 leukocytes /samples were enumerated by heterophil to lymphocyte separation. The heterophil to lymphocyte (H/L) proportion was calculated by dividing the number of heterophils by the number of lymphocytes (Gross and Siegel, 1999).

Statistical analysis

The study was conducted in a completely randomized design with five treatments. Statistical analysis was performed using the GLM method of SAS (SAS Institute, 1999), and Duncan's new multiple range tests were applied to determine significant differences (P < 0.05) between treatments

Results

The performance of laying hens is summarized in Table 2. In hens treated with MTEO (200 mg/kg) and NEO (100 and 200 mg/kg), egg weight was significantly more throughout the test time and the lowest egg weight was observed in hens fed with 100 mg/kg of MTEO (P < 0.05). Compared with the control and 100 mg/kg of NEO groups, dietary supplementation with 200 mg/kg of MTEO led to a higher egg production rate (P < 0.05). No significant difference was observed in egg mass, FI, and FCR in laying hens with the addition of MTEO and NEO to their diet.

Table 2. Effect of dietary levels of MTEO and NEO on laying performance

Treatment [1]	Egg weight (g)	Egg production rate (%)	Egg mass (g/d)	FI [2] (g/hen/d)	FCR [3] (kg of feed/kg of egg)
Control	54.87^{ab}	75.44^{b}	41.40	89.30	2.15
$MTEO_{100}$	53.11^{b}	77.88^{ab}	41.36	86.09	2.08
$MTEO_{200}$	57.30^{a}	78.36^{a}	44.91	87.01	1.94
NEO_{100}	56.44^{a}	75.87^{b}	42.84	86.57	2.02
NEO_{200}	56.26^{a}	76.47^{ab}	43.04	88.53	2.06
SEM	0.735	0.921	2.702	0.068	0.735
P-value	0.045	0.047	0.086	0.912	0.318

[a, b] Mean values in the same row with different superscript letters were significantly different (P < 0.05).
SEM: standard error of the means, MTEO: milk thistle essential oils, NEO: nettle essential oils
[1] $MTEO_{100}$= 100 mg MTEO per kg of diet, $MTEO_{200}$= 200 mg MTEO per kg of diet, NEO_{100}= 100 mg NEO per kg of diet, NEO_{200}= 200 mg NEO per kg of diet. [2] FI= Feed intake. [3] FCR= Feed conversion ratio.

Egg quality characteristics including shell thickness, shell breaking strength, HU, and yolk color did not vary among treatment classes (Table 3). The serum concentration of glucose, triglycerides, cholesterol, albumin, and total protein wasnot influenced by dietary concentrations of MTEO and NEO (Table 4).

The effect of dietary supplementation of MTEO and NEO on some hematological parameters of the laying hens is presented in Table 5. Dietary levels of MTEO and NEO showed no influence on lymphocyte count. The H/L ratio and heterophil count were significantly influenced by dietary treatments. In laying hens fed diets supplemented with 100 and 200 mg/kg of MTEO, significant decreases were observed in the percentage of heterophils compared with the control (P < 0.05). Moreover, the use of 200 mg/kg of MTEO decreased the heterophil percentage compared to 200 mg/kg of NEO treatment. There was a significant decrease in H/L ratio of hens given 200 mg/kg of MTEO compared with control and 200 mg/kg of NEO (P < 0.05).

Table 3. Effect of dietary levels of MTEO and NEO on egg quality parameters in laying hens

Treatment [1]	Shell thickness (mm)	Shell breaking strength (kg/cm^2)	HU (score)	Egg yolk color
Control	0.55	1.13	85.90	7.50
$MTEO_{100}$	0.55	1.32	87.66	6.35
$MTEO_{200}$	0.58	1.40	89.03	7.60
NEO_{100}	0.55	1.28	89.56	6.45
NEO_{200}	0.57	1.33	89.65	6.40
SEM	0.016	0.124	2.593	0.471
P-value	0.561	0.700	0.858	0.185

SEM: standard error of the means, MTEO: milk thistle essential oils, NEO: nettle essential oils.
[1]$MTEO_{100}$= 100 mg MTEO per kg of diet, $MTEO_{200}$= 200 mg MTEO per kg of diet, NEO_{100}= 100 mg NEO per kg of diet, NEO_{200}= 200 mg NEO per kg of diet.

Table 4. Effect of dietary levels of MTEO and NEO on serum parameters in laying hens

Treatment[1]	Glucose (mg/dL)	Triglyceride (mg/dL)	Cholesterol (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)
Control	186.66	1272.67	168.00	5.10	3.06
$MTEO_{100}$	188.16	1203.00	158.00	5.06	2.85
$MTEO_{200}$	180.50	1127.67	156.00	4.70	2.70
NEO_{100}	177.16	1164.33	164.33	4.98	2.98
NEO_{200}	173.66	1127.33	172.50	5.11	2.93
SEM	5.957	47.922	5.921	0.162	0.091
P-value	0.437	0.212	0.298	0.407	0.090

SEM: standard error of the means, MTEO: milk thistle essential oils, NEO: nettle essential oils
[1]$MTEO_{100}$= 100 mg MTEO per kg of diet, $MTEO_{200}$= 200 mg MTEO per kg of diet, NEO_{100}= 100 mg NEO per kg of diet, NEO_{200}= 200 mg NEO per kg of diet.

Table 5. Effect of dietary levels of MTEO and NEO on hematological parameters in laying hens

Treatment[1]	Heterophil (%)	Lymphocyte (%)	H/L
Control	29.33^{a}	74.50	0.39^{a}
$MTEO_{100}$	24.00^{bc}	73.33	0.32^{ab}
$MTEO_{200}$	22.50^{c}	75.00	0.30^{b}
NEO_{100}	26.83^{abc}	72.83	0.36^{ab}
NEO_{200}	28.16^{ab}	72.66	0.38^{a}
SEM	1.531	1.782	0.022
P-value	0.023	0.873	0.037

$^{a, b, c}$ Mean values in the same row with different superscript letters were significantly different ($P < 0.05$).
SEM: standard error of the means, MTEO: milk thistle essential oils, NEO: nettle essential oils.
[1]$MTEO_{100}$= 100 mg MTEO per kg of diet, $MTEO_{200}$= 200 mg MTEO per kg of diet, NEO_{100}= 100 mg NEO per kg of diet, NEO_{200}= 200 mg NEO per kg of diet.

Discussion

Milk thistle is famous for appreciating its impact on liver health in chickens. In the current experiment, in laying hens, dietary MTEO increased egg weight and egg production, a finding which agrees with previous results that reported dietary supplementation of milk thistle increased egg production (Hashemi Jabali *et al*., 2018). In line with the same works, Ather (2000) reported that adding EO in the broiler breeder diet causing in notable improvements in egg production. There is a report, which confirms the beneficial effects of dietary additive of EO of thyme, sage, and rosemary on egg weight in white layer strain (Bolukbasi *et al*., 2008). According to the reports accessible, improvement of layer diets with EO may influence yield index in laying hens (Bolukbasi *et al*., 2008, 2009) and such improvements may be attributed to stimulation of the internal secretions in the small enteric mucosa, liver, and pancreas and thus facilitation in digestion process (Brenes and Roura, 2010). Our findings, however, is not according to the result reported by Poudel and Khanal (2011), who stated that dietary additives of layers diets with nettle increased the average weekly egg production. The positive effect of nettle on egg production may be appropriate to the presence of high amounts of vitamins, minerals, and non-specific immunomodulators in the medicinal plant that activates the gene accountable for egg laying. Regarding the effects of EO on layer performance, Bolukbasi *et al*. (2008) stated that dietary thyme oil had no significant effect on FCR. Our results also disagree with Quarantelli *et al*. (2009) findings, who found dietary administration of silymarin in laying hen diets significantly diminished FI. In the present study, the FCR of hens was not affected by adding MTEO and NEO to the diet. The lack of influence of pharmaceutical plants on feed efficiency may be in part appertaining to laying hens as adult birds have an advanced and moderated digestive system, make bettering immunity, and raised resistance to enteric disorders in comparison to younger birds. On the contrary, Williams and Losa (2001) stated that medicinal EO has an affirmative impact on the digestive systems of the animal. Many studies suggested that these factors could be, in part, because of the increased production of digestive enzymes such as amylase and pancreatic lipase (Williams and Losa, 2001; Ramakrishna *et al*., 2003). Increased secretion of digestive enzymes can be one of the reasons for increased production in birds receiving EO-added diets. Another agent that could be proof of the positive impacts of certain medicinal plants is their essential fatty acid content. Many herbs provide considerable magnitudes of linolenic, linoleic, and arachidonic acids that are essential for appropriate yield. (Brenes and Roura, 2010). Previous studies reported that the addition of milk thistle to an aflatoxin-contaminated diet improved FCR in laying hens. Kralik *et al*. (2015) offered that dietary improvement of MTEO improved live weight and FCR in broilers (Tedesco *et al*., 2004). It is essential to note silymarin can assist in the antioxidant defense by free radical scavenging and thus improve health conditions and performance (Surai, 2015). Moco *et al*., 2012 reported that polyphenols create antioxidant defense in the lower enteric and can change gut flora. Polyphenols, which include silybin, are broadly metabolized by enteric bacteria into an intricate series of final products that influence the act bionomics of symbiotic partners that can change the host physiology (Moco *et al*., 2012).

In our study, HU, the main index of interior egg quality, was not affected by the dietary additive of MTEO and NEO. Bozkurt *et al*. (2012) showed that the additive of an EO mixture into a diet for laying hens did not remarkably change yolk weight, HU, and albumen height but reduced the relative weight of albumen. Egg mass, egg weight, and eggshell thickness were notably affected by an essential oil mixture additive (Olgun, 2016). Poudel and Khanal (2011) reported that nettle could increase calcium content in eggs. In addition, nettle raises the thickness of eggshells, which can later decrease the broken egg series and further breakage casualties. One of the most significant indicators in evaluating the quality of eggs is yolk color because pigment intensity in the yolk affects egg organoleptic indices, and may influence consumer satisfaction. Feed supplements and raising procedures (e.g., free range) are the main agents influencing egg yolk pigmentation. Lokaewmanee *et al*., 2009 showed that consumers are more inclined to eggs with darkish-colored yolks. Changes noticed in yolk color are related mainly to the components used in diets. Nettle supplementation in layers diets improved egg yok color (Loetscher *et al*., 2013).

In this research, serum concentrations of triglycerides, cholesterol, glucose, and total protein were not influenced by nettle and milk thistle. According to the previous literature, silymarin supplementation did not affect urea nitrogen amount, protein, serum glucose, calcium, bilirubin, phosphorus, and aspartate aminotransferase (AST) (Tedesco *et al*. 2004). Tuorkey *et al*., 2015 stated that silymarin in diabetic rats remarkably decreased plasma levels of cholesterol, triglycerides, and AST. Silymarin is believed to function essentially, via anti-inflammatory and antioxidant features and stimulates liver cell modification (Vargas-Mendoza *et al*., 2014).

In poultry production, improved immunity response is of prime importance to prevent the harmful effects of pathogens and stressors (Thyagarajan *et al*., 2002; Wilasrusmee *et al*., 2002). According to previous reports, milk thistle raises lymphocyte multiplication which is related to increased interleukin (IL)-4, interferon-gamma, and IL-10 cytokines. (Wilasrusmee *et al*., 2002). To a report by Acamovic and Brooker (2005) medicinal plants that are rich in flavonoids raise the acting of vitamin C, function as antioxidants, and may so make better immune act. Antioxidant properties of some medicinal herbal products, such as extracts and EO, have been believed to have a function in the expansion of immune response in birds by defending cells from oxidative stress and increasing the action and multiplication of these cells (Sun *et al*., 1983). Nettle feeding stimulates immune response in laying hens by lymphocyte proliferation (Poudel and Khanal, 2011). Silymarin, as an extract of the milk thistle seeds, is a complex of four flavonolignans with intense free-radical scavenging and antioxidant features (Wu *et al*., 2008). Hematological indices are commonly related to health status, so they are considered an index of the pathological, physiological, and nutritional condition of an animal. They also have the possibility of being applied to explain the effect of dietary agents and supplements provided in the diet. For instance, leucocytes are well known to raise during infection due to they are one of the first lines of protection in the body (Ganong, 1999). The ratio of H/L has been used as a physiological indicator of stress in the assessment of chicken reaction to a new habitat and different stressors (Maxwell, 1993). In the present research, the lower H/L observed in layer hens fed diets, including MTEO, implies the positive influence of MTEO on reducing stress.

Conclusion

As a result, the finding of this research proposes that the supplementation of diets for laying hens with 200 mg/kg of MTEO could positively affect egg production and immunity response.

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