Lonicera flos and *Cnicus japonicus* extracts improved egg quality partly by modulating antioxidant status, inflammatory-related cytokines and shell matrix protein expression of oviduct in laying hens

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ABSTRACT This study was conducted to investigate the effects of *Lonicera flos* and *Cnicus japonicus* extracts (**LCE**) on the laying performance, egg quality, morphology, antioxidant status, inflammatory-related cytokines, and shell matrix protein expression of oviduct in laying hens. A total of 1,728 Roman Pink laying hens aged 73-wk-old were randomly assigned into 4 groups (18 replicates/group, 24 layers/replicate) fed basal diets supplemented with 0, 300, 500, and 1,000 mg of LCE per kg of diet, respectively. The trial lasted for 11 wk, including 2-wk adjustment period and 9-wk testing period. The results indicated that laying hens fed diets supplemented with LCE linearly increased egg weight, yolk color and shell thickness at wk 78 and albumen height, Haugh unit and shell thickness at wk 83 ($P < 0.05$). At wk 78, LCE groups linearly affected the hydrogen peroxide content in magnum ($P < 0.05$) and 300 mg/kg LCE groups had the highest catalase activity in isthmus ($P < 0.05$). At wk 83, LCE groups linearly reduced ($P < 0.05$) hydrogen peroxide content in the magnum and isthmus and malondialdehyde content in the uterus whereas increased catalase activity in isthmus ($P < 0.05$). Furthermore, LCE levels quadratically affected glutathione peroxidase activity in isthmus at wk 83 ($P < 0.05$). At wk 78, the mRNA expressions of inducible nitric oxide synthase and interferon-γ in isthmus and ovalbumin and ovocleidin-116 in uterus had linear effects in response to LCE levels ($P < 0.05$) and 1,000 mg/kg LCE group had the lowest mRNA expression of interleukin-6 in magnum ($P < 0.05$). At wk 83, LCE supplementation linearly decreased the mRNA expression of interleukin-1β, interferon-γ and tumor necrosis factor-α in magnum and tumor necrosis factor-α and inducible nitric oxide synthase in uterus ($P < 0.05$). It is concluded that LCE improved egg quality partly by modulating antioxidant status, inflammatory-related cytokines and shell matrix protein expression of oviduct in laying hens.

Key words: antioxidant status, *Cnicus japonicus*, inflammatory cytokine, laying hen, *Lonicera flos*

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INTRODUCTION

In poultry, the formation of egg occurs in the fallopian tubes, involving complex regulation of various genes and biological pathways (Sah et al., 2018; Sah and Mishra, 2018). Oviduct infection of laying hens, such as *Salmonella* and *Escherichia coli*, can cause damage to the oviduct lining and seriously cause a decrease in egg production and poor egg quality (Burnham et al, 2002; Fan et al, 2014). The quality of eggs is closely related to the health of the fallopian tube (Carillon et al., 2016). Therefore, the good health of the oviduct is very important for the breeders.

The use of antibiotics in poultry production is restricted because it can increase microbial resistance which can be passed to infectious microorganisms in humans (Li et al, 2006). It is important to explore alternatives to antibiotics to improve the health and production performance of the poultry industry.

Lonicerae flos ("Shanyinhua" in Chinese, the dried flower bud or newly bloomed flower of *Lonicera hypoglauca*) is a traditional Chinese medicine, whose main active ingredients were chlorogenic acid (**CGA**, a kind of polyphenols) (Liao et al., 2013), and saponins (Gong et al., 2016). Modern pharmacologic studies have

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confirmed the antiviral, antibacterial and anti-inflammatory activities of *L. flos* (Kang et al., 2004; Kao et al., 2015; Shi et al., 2016). *Cnicus japonicus* ("Daji" in Chinese, the dry aboveground part or root of a plant), like milk thistle, belongs to the genus thistle in the composite family and has hemostatic, antibacterial and antitumor effects (Fu et al., 2003). It has been reported that the main compounds isolated from thistle are flavonoids and flavonoid glycosides, long chain alkynols, volatile oils, sterols, and other classes (Zhi et al., 2001). As natural phenolic compounds, flavonoids have strong antioxidant properties due to the presence of aromatic hydroxyl group (Vessal et al., 2010). As scavengers of reactive oxygen species and nitrogen, flavonoids inhibit peroxidation (Bors and Saran, 1987; Bors et al., 1990) and also have a protective effect against oxidative stress (Duthie and Crozier, 2000). Accordingly, addition of the extracts from *L. flos* and *C. japonicus* may exert active roles in protecting the oviduct in poultry.

More and more research results supported the important role of plant-derived products such as flavonoids and polyphenols in antioxidant and anti-inflammatory functions in animals (Cheng et al., 2014; Liu et al., 2021; Guerrini and Tedesco, 2022; Khazaei et al., 2022). However, few literature is available about the effects of *L. flos* or *C. japonicus* on laying hens and it is not clear whether addition of *L. flos* and *C. japonicus* extracts (**LCE**) can regulate the health of fallopian tubes in laying hens, thereby helping to protect them from pathogen infection. Therefore, the present study was to evaluate the effects of LCE on the laying performance, egg quality and tissue morphology, inflammatory cytokines and shell matrix protein gene expression of oviduct in laying hens, indicating the possible regulatory effects of LCE on the fallopian tube status of laying hens.

MATERIALS AND METHODS

All experimental procedures were conducted in accordance with Hubei Provincial Regulations for Laboratory Animals (011043145-029-2013-000009), and were approved by the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Number: WPU202106003).

Birds, Diets, and Management

A total of 1,728 Roman Pink laying hens aged 73-wk-old were randomly assigned into 4 groups with 18 replicates of 24 layers, which were fed basal diets supplemented with 0, 300, 500, and 1,000 mg of LCE (Centre (Inner Mongolia) Technology Co., Ltd.) per kg of diet, respectively. The basal diet was formulated according to the recommendation (NY/T33-2004) for laying hens. The composition and nutrient levels of basal diet were shown in Table 1. Four laying hens were allotted to 1 cage (57 cm × 57 cm × 47 cm, equipped with 1 nipple drinkers and 1 feeder). The temperature and relative humidity in house were 22 ± 2 °C and 60 to 70%, respectively, with a 16:8 L:D light program used during the trial period. The trial lasted for 11 wk, including 2-wk adjustment period and 9-wk testing period.

Table 1. The composition and nutrient levels of basal diet for laying hens.

Ingredients (%)	Content	Nutrient levels (%)[2]	Content
Corn	66.21	Metabolizable energy (MJ/kg)	11.31
Soybean meal	21.00	DM	88.69
Wheat bran	2.00	CP	14.90
Dicalcium phosphate	2.00	Calcium	3.46
Limestone	8.00	Available phosphorus	0.44
Salt	0.30	Methionine	0.35
DL-Methionine	0.10	Lysine	0.80
L-Lysine	0.10	Methionine + cysteine	0.62
50% choline chloride	0.10		
Premix[1]	0.19		
Total	100.00		

[1]The premix provided the following per kilogram of the diet: vitamin A 9000 IU; vitamin D_3 3500 IU; vitamin E 20 IU; vitamin K_3 2 mg; vitamin B_1 5 mg; vitamin B_2 7 mg; vitamin B_6 5 mg; vitamin B_{12} 0.02 mg; folic acid 1 mg; biotin 0.2 mg; niacin 45 mg; calcium pantothenate 10 mg; I 1 mg; Se 0.3 mg.

[2]CP were analyzed value. The other nutrient levels were calculated. Amino acid levels are expressed on a total basis.

Sample Collection

At the end of wk 78 and wk 83, 54 intact eggs per group (3 eggs per replicate) were randomly collected for egg quality measurements. Ten birds per group were randomly selected and blood collected from the wing vein were centrifuged for 10 min (3,000 × g, 4 °C) to collect plasma and stored at -80°C until further assay. Then, oviduct tissues were collected 16 h after oviposition when a calcifying egg was in the uterus and the laying hens were euthanized by cervical dislocation. Approximately 1 cm^2 of oviduct segments (magnum, isthmus, and uterus) were cut from the same position and fixed into 4% neutral paraformaldehyde solution for oviduct morphology analysis. Another magnum, isthmus and uterus segments were sampled and stored at -80°C for the assay of antioxidant status and gene expression.

Laying Performance

The egg production and egg weight in each replicate were recorded daily, and the residual feed consumption was recorded weekly. Egg production, average egg weight, egg mass, feed intake, and feed conversion ratio (**FCR**, feed/egg) were calculated at the end of wk 78 and 83. All the calculation was conducted as described by Xiao et al. (2019).

Egg Quality Determination

Albumen height, Haugh unit, yolk color and egg weight were measured with an egg analyzer (ORKA Food Technology Ltd., Ramat Hasharon, Israel). The height and width of egg was measured with a vernier caliper (Robotmation Co., Ltd. Tokyo, Japan) and shape

index was calculated according to the formula: shape index = height / width. The eggshell thickness was calculated by averaging the thickness of 3 different points (air cell, sharp end and any side of the equator) measured using vernier calipers. Eggshell strength was measured with an egg force reader (ORKA Food Technology Ltd., Ramat Hasharon, Israel).

Oviduct Morphology Analysis

After stained with hematoxylin and eosin, the oviduct samples (magnum, isthmus, and uterus) were examined under a DM3000 microscope (Leica, Wetzlar, Germany) for morphometry analysis (Scale bar: 500 μm) including primary villus length, secondary villus length and primary villus area in magnum and villus length in isthmus (Shen et al., 2014). The intervillous spaces in uterus were averaged for 3 to 5 adjacent villi spaces (averaged from 10 values between 2 adjacent villi) in each slice (Li et al., 2022). The measurements of oviduct morphology were made using Image-Pro software (Media Cybernetics, Rockville, MD).

Antioxidant Status Assay

Oviduct segments were homogenized in ice-cold phosphate-buffered saline at a ratio of 1:10 (g/mL) and centrifuged at 3 000 × g, 4 °C for 10 min. The supernatants were collected for antioxidant status analysis. The activities of glutathione peroxidase (**GSH-Px**), total superoxide dismutase (**T-SOD**) and catalase (**CAT**), the contents of malondialdehyde (**MDA**) and hydrogen peroxide ($\mathbf{H_2O_2}$) in the plasma and supernatants were measured using assay kits (Nanjing Jiancheng Institute of Bioengineering, Nanjin, China).

RNA Isolation and Quantitative Real-Time PCR

The expressions of interleukin-β (**IL-1β**), interleukin-6 (**IL-6**), interferon-γ (**IFN-γ**), tumor necrosis factor-α (**TNF-α**), inducible nitric oxide synthase (**iNOS**) clusterin, ovocleidin-116 (**OC-116**), ovalbumin (**OVA**) and ovotransferrin mRNA in magnum, isthmus and uterus were determined on a real-time PCR system (ABI 7500; Applied Biosystems, Foster City, CA) following the protocol of SYBR Premix Ex Taq kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) as previously described (Guo et al., 2017; Guo et al., 2020). First, the total RNA was extracted from approximately 20 mg of samples using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of total RNA were quantified by measuring its optical density at 260 and 280 nm with a NanoDrop spectrophotometer (ND-2000, Thermo Scientific). The agarose gel electrophoresis was used to verify RNA integrity. Reverse transcription was performed from 1 μg total RNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara) according to the manufacturer's instructions. The oligonucleotide primers are listed in Table 2. The PCR was performed with the following thermal procedure: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. There were 10 samples for each group, and each sample was performed in triplicate. The method $2^{-\Delta\Delta CT}$ and the internal reference genes (β-actin) were used to calculate the mRNA level of each target gene (expressed as the relative values to the control group).

Statistical Analyses

All experimental data were presented as means with SEM and analyzed statistically by one-way ANOVA using SPSS software (version 21.0; IBM Inc., NY). Polynomial contrasts were used to determine the linear and quadratic effects of dietary LCE supplementation. Differences among groups were analyzed by Duncan's multiple comparison tests. Values of $P < 0.05$ were considered significant. All the graphs were made using GraphPad Prism 8.4.3.

RESULTS

Laying Performance

The effects of dietary LCE supplementation on laying performance of laying hens were shown in Table 3. At the period of wk 75 to 78 and 79 to 83, LCE had no

Table 2. Primers used for real-time quantitative fluorescence PCR analysis.

	Primer (5′→3′)		
Gene	Forward	Reverse	Accession no.
IL-1β	ATGAACGGCAAGC TTGGAGCTG	TCCAAGCACACC TCTCTTCCATC	AJ009800
IL-6	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT	AJ309540
IFN-γ	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	NM_205149
TNF-α	GAGCGTTGACTTGGCTGTC	AAGCAACAACCAGCTATGCAC	NM_204267
iNOS	CAGCTGATTGGGTGTGGAT	TTTCTTTGGCCTACGGGTC	U46504
CLU	GCTTCCACCGCCTTC	CACTCTCGCACTCCC	NM_204900.1
OVA	GCTATGGGCATTACTGACG	TGCTGACCCTCACCTCT	NM_205152.2
OVOT	ATTGCTGCTGAGATTTATG	TTCTATGCCTTCCCACT	NM_205304.1
OC-116	GCTTTTGATGAGACTGGACGAG	AGCGGGTAGCGACAACATC	AF148716.3
β-actin	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA	NM_205518

Abbreviations: CLU, clusterin; IFN-γ, interferon-γ; IL-1β, interleukin-β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; OC-116, ovocleidin-116; OVA, ovalbumin; OVOT, ovotransferrin; TNF-α, tumor necrosis factor-α.

Table 3. Effects of *Lonicera flos* and *Cnicus japonicus* extract on the laying performance of Roman Pink hens from 75 to 83 wk of age (n = 18).

Items	LCE (mg/kg)				SEM	*P*-value		
	0	300	500	1000		Combined	Linear	Quadratic
75−78 wk								
FI (g/hen/d)	112.1	112.1	111.2	112.6	0.267	0.285	0.783	0.199
Egg mass (g/hen/d)	48.7	48.6	49.5	49.7	0.485	0.809	0.378	0.883
FCR (g of feed/g of egg)	2.32	2.32	2.25	2.28	0.022	0.714	0.423	0.782
Egg production (%)	76.89	76.30	78.05	78.15	0.792	0.814	0.446	0.830
Egg weight (g/egg)	63.4	63.7	63.5	63.7	0.180	0.870	0.651	0.795
79−83 wk								
FI (g/hen/d)	111.6	112.2	111.0	112.3	0.271	0.280	0.699	0.491
Egg mass (g/hen/d)	47.9	47.8	48.4	49.8	0.488	0.444	0.151	0.442
FCR (g of feed/g of egg)	2.35	2.36	2.30	2.27	0.023	0.470	0.162	0.605
Egg production (%)	75.41	74.92	76.19	77.97	0.806	0.568	0.222	0.487
Egg weight (g/egg)	63.5	63.9	63.6	64.0	0.188	0.801	0.551	0.932

Abbreviations: FI, Feed intake; FCR, Feed conversion ratios; LCE, *Lonicera flos* and *Cnicus japonicus* extract.

significant effects on the egg production, egg weight, egg mass, feed intake, and FCR of aged Roman Pink laying hens ($P > 0.05$).

Egg Quality

As shown in Table 4, compared to the control group, laying hens fed diets supplemented with LCE had the increased yolk color and decreased yolk ratio at wk 78 ($P < 0.05$). Moreover, egg weight and shell thickness linearly increased with increasing LCE levels ($P < 0.05$) and LCE levels quadratically affected shell ratio at wk 78 ($P < 0.05$). At wk 83, albumen height, Haugh unit and shell thickness linearly increased with increasing LCE levels ($P < 0.05$) whereas the yolk color linearly decreased with increasing LCE levels ($P < 0.05$).

Oviduct Morphology

The morphology of oviduct segments was shown in Figure 1. Under the microscope, neither control group nor the LCE supplementation had villi shedding or inflammation-related symptoms. Accordingly, it was showed that LCE supplementation had no significant effect on the primary villus length, secondary villus length and primary villus area in magnum, villus length in isthmus and the intervillous spaces in uterus.

Antioxidant Status

At wk 78, LCE groups linearly affected the H_2O_2 content in magnum ($P < 0.05$) and 300 mg/kg LCE groups had the highest CAT activity in isthmus ($P < 0.05$) (Table 5). At wk 83 (Table 6), LCE groups linearly reduced H_2O_2 content in the magnum and isthmus ($P < 0.05$) and MDA content in the uterus ($P < 0.05$). In

Table 4. Effects of *Lonicera flos* and *Cnicus japonicus* extract on the egg quality of Roman Pink hens at wk 78 and 83 (n = 54).

Items	LCE (mg/kg)				SEM	*P*-value		
	0	300	500	1,000		Combined	Linear	Quadratic
78 wk								
Egg weight (g)	65.19b	66.71[a]	66.99[a]	67.72[a]	0.236	0.001	<0.001	0.392
Shape index	1.34	1.34	1.33	1.33	0.003	0.372	0.078	0.972
Albumen height (mm)	4.46	4.79	4.78	4.72	0.099	0.612	0.589	0.761
Haugh unit	59.08	61.03	60.19	59.10	1.089	0.907	0.761	0.797
Yolk color	5.70[c]	7.53[a]	5.16[c]	6.33[b]	0.121	<0.001	<0.001	<0.001
Yolk ratio (%)	29.15[a]	28.73[ab]	28.16[bc]	27.53[c]	0.134	<0.001	0.916	0.944
Shell ratio (%)	10.55[a]	10.32[ab]	10.13[b]	10.5[a]	0.048	0.009	0.401	0.002
Shell thickness (mm)	0.34[b]	0.34[b]	0.35[b]	0.36[a]	0.002	<0.001	<0.001	0.100
Shell strength (N)	42.73	42.22	41.00	42.67	0.506	0.597	0.754	0.283
83 wk								
Egg weight (g)	70.17	70.86	70.96	70.24	0.248	0.567	0.891	0.159
Shape index	1.33	1.35	1.35	1.34	0.004	0.278	0.841	0.055
Albumen height (mm)	4.67[b]	4.87[b]	5.50[ab]	5.81[a]	0.148	0.021	0.002	0.850
Haugh unit	57.02[c]	59.22[bc]	66.67[a]	64.98[ab]	1.190	0.010	0.003	0.406
Yolk color	5.95[a]	5.85[a]	5.07[b]	5.31[b]	0.083	<0.001	<0.001	0.291
Yolk ratio (%)	26.47	26.66	26.54	26.54	0.123	0.951	0.897	0.810
Shell ratio (%)	9.80	9.91	9.75	9.75	0.049	0.664	0.956	0.978
Shell thickness (mm)	0.34[d]	0.36[c]	0.38[b]	0.40[a]	0.003	<0.001	<0.001	0.580
Shell strength (N)	37.84	39.90	41.09	41.25	0.586	0.146	0.031	0.400

Abbreviation: LCE, *Lonicera flos* and *Cnicus japonicus* extract.
[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

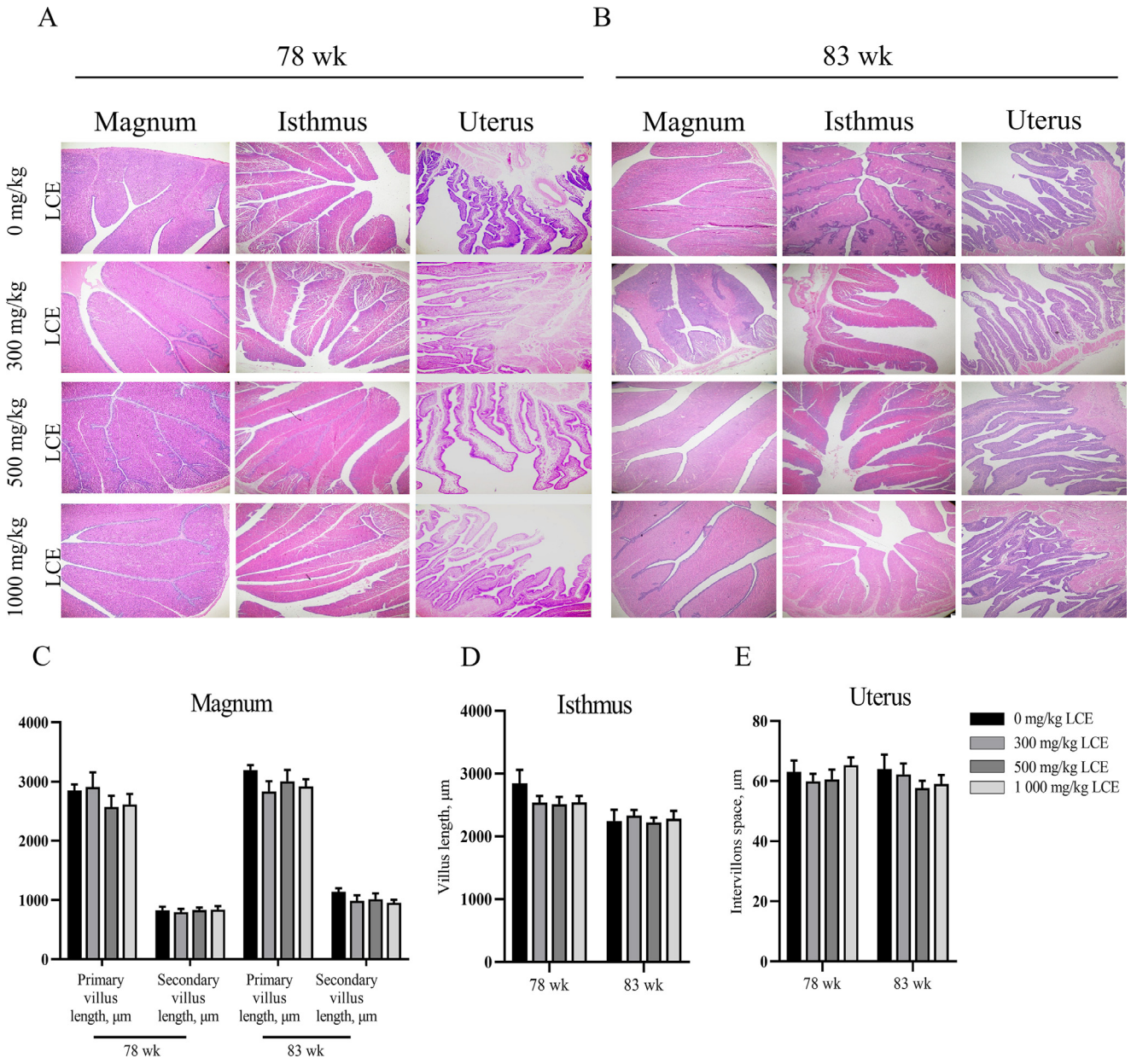

Figure 1. The morphology of fallopian tube in laying hens and measurements of primary villus length, secondary villus length and primary villus area in magnum, villus length in isthmus and intervillous space in uterus (Scale bar: 500 μm). Data are presented as means and SEM. Values with no letters or the same superscripts are not significantly different, whereas those with different superscript letters are significantly different ($P < 0.05$). LCE, *Lonicera flos* and *Cnicus japonicus* extract.

addition, CAT activity in isthmus linearly increased but T-SOD activity in magnum decreased at wk 83 ($P < 0.05$), and LCE levels quadratically affected GSH-Px activity in isthmus at wk 83 ($P < 0.05$). Moreover, LCE supplementation had significant effects on CAT, GSH-Px and T-SOD activity in uterus at wk 83 ($P < 0.05$).

Expression of Inflammatory Genes mRNA

At wk 78 (Table 7), the mRNA expressions of iNOS in magnum and IL-6 in uterus had quadratical effects in response to LCE levels ($P < 0.05$) and 1,000 mg/kg LCE group had the lowest mRNA expression of IL-6 in magnum and iNOS in isthmus ($P < 0.05$). Furthermore, the mRNA expression of IFN-γ in isthmus linearly decreased with increasing LCE levels at wk 78 ($P < 0.05$). At wk 83 (Table 8), LCE supplementation linearly decreased ($P < 0.05$) the mRNA expression of IL-β, IFN-γ, and TNF-α in magnum whereas increased ($P < 0.05$) the mRNA expression of iNOS in isthmus. In addition, 500 mg/kg LCE supplementation had the lowest mRNA expression of TNF-α and iNOS in uterus at wk 83 ($P < 0.05$).

Expression of Shell Matrix Protein mRNA

The relative mRNA expression levels of shell matrix protein in uterus were shown in Table 9. The mRNA expression levels of OVA and OC-116 linear increased in response to LCE treatments in uterus at wk 78 ($P < 0.05$).

Table 5. Effects of *Lonicera flos* and *Cnicus japonicus* extract on the antioxidant status in the magnum, isthmus and uterus of laying hens at wk 78 (n = 10).

	LCE (mg/kg)					*P*-value		
Items	0	300	500	1,000	SEM	Combined	Linear	Quadratic
Magnum								
CAT (U/mL)	6.65	6.69	6.47	6.75	0.307	0.949	0.841	0.559
GSH-Px (μmol/L)	82.87	90.62	93.34	91.44	4.135	0.840	0.853	0.992
H_2O_2 (mmol/L)	11.63^{ab}	12.49^{a}	13.49^{a}	9.54^{b}	0.488	0.024	0.018	0.208
MDA (nmol/L)	4.14	3.94	4.64	4.74	0.156	0.202	0.477	0.275
T-SOD (U/mL)	110.92	101.46	108.04	120.83	3.732	0.316	0.324	0.771
Isthmus								
CAT (U/mL)	14.72^{b}	26.84^{a}	9.67^{b}	16.90^{b}	1.852	0.002	0.442	0.420
GSH-Px (μmol/L)	220.65	303.76	232.5	238.55	21.78	0.506	0.319	0.247
H_2O_2 (mmol/L)	15.24	19.2	15.18	17.68	1.105	0.484	0.760	0.750
MDA (nmol/L)	6.96	8.37	5.42	6.04	0.429	0.065	0.125	0.628
T-SOD (U/mL)	439.31	613.28	472.70	477.68	37.21	0.325	0.940	0.264
Uterus								
CAT (U/mL)	4.17	3.83	4.54	3.85	0.231	0.683	0.907	0.721
GSH-Px (μmol/L)	197.81	201.23	198.31	191.68	3.804	0.852	0.545	0.528
H_2O_2 (mmol/L)	17.10	14.71	14.23	15.12	0.443	0.099	0.096	0.061
MDA (nmol/L)	1.13	1.16	1.01	1.00	0.078	0.861	0.455	0.903
T-SOD (U/mL)	301.72	266.44	300.02	297.01	6.557	0.233	0.732	0.220

Abbreviations: CAT, catalase; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; LCE, *Lonicera flos* and *Cnicus japonicus* extract; MDA, malondialdehyde; T-SOD, total superoxide dismutase.

[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

DISCUSSION

Previous study has indicated that *L. hypoglauca* extracts could enhance antioxidant status in inflammation-related diseases due to the bioactive component CGA in *L. hypoglauca* (Liao et al., 2013). As a kind of polyphenols widely existing in natural plants, CGA has many biological functions, such as antioxidation, anti-inflammation, bacteriostasis, antivirus and anticancer (Ruan et al., 2016; Rui et al., 2017; Lee and Lee, 2018; Li et al., 2018; Naveed et al., 2018). Study have showed that dietary supplementation with 18 g/kg *Eucommia ulmoides* leaves (CGA: ≥3.5%) could significantly increase egg production, egg weight and decrease feed conversion efficiency (Kang et al., 2018). Little research has been done on *C. japonicus* in laying hens whereas milk thistle is the most studied thistle. Dietary administration of milk thistle meal significantly reduced FCR in the second 35 d and whole experimental periods (Hashemi Jabali et al., 2017). However, in the present study, no significant effects of different concentrations of LCE on the laying performance of laying hens were observed throughout the trial period. This may be related to the difference of breed, week age, *L. flos* and *C. japonicus* concentration and test period of laying hens.

Table 6. Effects of *Lonicera flos* and *Cnicus japonicus* extract on the antioxidant status in the magnum, isthmus and uterus of laying hens at wk 83 (n = 10).

	LCE (mg/kg)					*P*-value		
Items	0	300	500	1,000	SEM	Combined	Linear	Quadratic
Magnum								
CAT (U/mL)	13.59	14.16	12.15	11.39	0.541	0.251	0.081	0.535
GSH-Px (μmol/L)	101.09	74.90	104.59	95.25	4.386	0.069	0.742	0.313
H_2O_2 (mmol/L)	6.46^{a}	5.87^{ab}	4.53^{bc}	4.40^{c}	0.277	0.011	0.002	0.643
MDA (nmol/L)	2.12	1.80	1.58	1.60	0.123	0.379	0.112	0.485
T-SOD (U/mL)	173.70^{a}	174.07^{a}	137.03^{b}	126.23^{b}	5.555	<0.001	<0.001	0.333
Isthmus								
CAT (U/mL)	5.75^{b}	7.57^{ab}	9.29^{a}	9.42^{a}	0.512	0.028	0.004	0.365
GSH-Px (μmol/L)	100.56^{b}	124.29^{b}	172.11^{a}	115.22^{b}	7.768	0.003	0.121	0.004
H_2O_2 (mmol/L)	10.62^{a}	6.69^{c}	8.69^{b}	5.78^{c}	0.429	<0.001	<0.001	0.432
MDA (nmol/L)	2.22	1.83	2.46	2.05	0.098	0.133	0.879	0.955
T-SOD (U/mL)	160.01	164.65	204.04	163.25	6.760	0.060	0.385	0.081
Uterus								
CAT(U/mL)	3.60^{a}	3.61^{a}	1.77^{b}	3.09^{a}	0.213	0.001	0.025	0.048
GSH-Px (μmol/L)	156.12^{a}	106.32^{b}	108.03^{b}	102.07^{b}	4.828	<0.001	<0.001	<0.001
H_2O_2 (mmol/L)	17.42	15.38	15.82	13.99	0.508	0.118	0.030	0.913
MDA (nmol/L)	2.28^{a}	2.15^{a}	1.92^{b}	1.71^{b}	0.053	<0.001	<0.001	0.608
T-SOD (U/mL)	320.46^{b}	352.61^{a}	303.30^{b}	274.39^{c}	6.659	<0.001	<0.001	0.003

Abbreviations: CAT, catalase; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; LCE, *Lonicera flos* and *Cnicus japonicus* extract; MDA, malondialdehyde; T-SOD, total superoxide dismutase.

[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 7. Effects of *Lonicera flos* and *Cnicus japonicus* extract on the expressions of inflammatory-related cytokines mRNA in the oviduct of laying hens at wk 78 (n = 10).

	LCE (mg/kg)					*P*-value		
Items	0	300	500	1,000	SEM	Combined	Linear	Quadratic
Magnum								
IL-1β	1.15	1.27	1.29	1.27	0.089	0.939	0.622	0.715
IL-6	1.06^{ab}	1.40^{a}	0.94^{b}	0.76^{b}	0.079	0.029	0.067	0.110
IFN-γ	1.14	0.84	0.70	0.61	0.075	0.073	0.011	0.471
TNF-α	1.07	0.88	0.91	0.75	0.058	0.256	0.073	0.935
iNOS	1.06^{c}	3.86^{a}	2.78^{b}	1.51^{c}	0.249	<0.001	0.874	<0.001
Isthmus								
IL-1β	1.31	0.93	1.13	1.35	0.147	0.738	0.817	0.337
IL-6	1.01	1.11	1.18	0.72	0.066	0.054	0.180	0.032
IFN-γ	1.11^{ab}	1.27^{a}	0.82^{ab}	0.69^{b}	0.084	0.044	0.022	0.343
TNF-α	1.13	1.22	0.87	0.94	0.114	0.693	0.406	0.976
iNOS	1.01^{a}	0.57^{b}	0.68^{b}	0.64^{b}	0.043	<0.001	0.003	0.006
Uterus								
IL-1β	1.08	1.10	1.07	1.16	0.064	0.964	0.732	0.811
IL-6	1.05^{b}	2.22^{a}	2.60^{a}	1.25^{b}	0.175	0.001	0.456	<0.001
IFN-γ	1.10	1.43	1.01	1.12	0.136	0.545	0.605	0.343
TNF-α	1.07	1.14	1.41	1.45	0.076	0.192	0.038	0.918
iNOS	1.05	1.40	1.38	1.53	0.083	0.162	0.049	0.571

Abbreviations: IL-1β, interleukin-β; IL-6, interleukin-6; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LCE, *Lonicera flos* and *Cnicus japonicus* extract; TNF-α, tumor necrosis factor-α.

[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

In the present study, it was found that yolk color increased with dietary supplementation of CGA at wk 78, which was analogous to the results of Kang et al. (2018). Because the carotenoids in the yolk are the determinants of the yolk color and depend on the nutrient content in the feed (Kljak et al., 2021), it is possible that LCE promotes the deposition of carotenoids in the egg yolk to enhance the yolk color. Albumen height and Haugh unit are important indicators of the protein quality and reflect the freshness of eggs (Qu et al., 2019). In the present study, dietary supplementation with LCE increased albumen height and Haugh unit at wk 83. Because plant polyphenols can increase the albumen height and Haugh unit owing to their antioxidative effects (Xie et al., 2019), the antioxidant capacity of CGA may be partly responsible for their effects on improving albumen quality. Eggshell strength and eggshell thickness are 2 primary indicators of eggshell quality, as they influence the storage and transportation stability of egg (Yuan et al., 2014). Our results showed that the addition of LCE significantly increased eggshell thickness at wk 78 and 83. Furthermore, the eggshell strength has no significant difference among all treatments. Inconsistent with our results, dietary addition 30 g/kg of milk thistle meal can improve eggshell strength and thickness (Gholamalian et al., 2022). After all, the study on milk thistle cannot fully represent whether the effect of thistle extract is the same.

Table 8. Effects of *Lonicera flos* and *Cnicus japonicus* extract on the expressions of inflammatory-related cytokines mRNA in the oviduct of laying hens at wk 83 (n = 10).

	LCE (mg/kg)					*P*-value		
Items	0	300	500	1000	SEM	Combined	Linear	Quadratic
Magnum								
IL-1β	1.23^{a}	0.63^{b}	0.44^{b}	0.19^{b}	0.110	0.007	0.001	0.362
IL-6	1.06	1.04	0.97	1.06	0.079	0.976	0.906	0.738
IFN-γ	1.17^{a}	0.92^{a}	0.49^{b}	0.53^{b}	0.078	0.006	<0.001	0.283
TNF-α	1.13^{a}	0.67^{b}	0.57^{b}	0.49^{b}	0.073	0.009	0.002	0.149
iNOS	1.04	0.98	1.20	0.94	0.071	0.591	0.904	0.495
Isthmus								
IL-1β	1.16	1.15	0.65	0.66	0.107	0.137	0.046	0.943
IL-6	1.02	0.77	1.03	0.87	0.056	0.298	0.718	0.711
IFN-γ	1.03	0.92	1.24	1.28	0.070	0.210	0.123	0.600
TNF-α	1.04	1.13	1.22	1.26	0.086	0.845	0.382	0.877
iNOS	1.08^{b}	1.28^{ab}	1.61^{ab}	1.75^{a}	0.096	0.046	0.006	0.857
Uterus								
IL-1β	1.04	1.17	0.97	0.94	0.072	0.692	0.471	0.578
IL-6	1.05	1.34	1.36	1.57	0.083	0.169	0.035	0.823
IFN-γ	1.14	0.73	0.87	0.93	0.080	0.422	0.528	0.160
TNF-α	1.04^{a}	0.62^{b}	0.61^{b}	0.71^{b}	0.049	0.005	0.014	0.004
iNOS	1.08^{b}	0.98^{b}	0.98^{b}	1.51^{a}	0.076	0.032	0.047	0.030

Abbreviations: IL-1β, interleukin-β; IL-6, interleukin-6; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LCE, *Lonicera flos* and *Cnicus japonicus* extract; TNF-α, tumor necrosis factor-α.

[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 9. Effects of *Lonicera flos* and *Cnicus japonicus* extract on the expressions of shell matrix protein mRNA in the uterus of laying hens at wk 78 and 83 (n = 10).

	LCE (mg/kg)					*P*-value		
Items	0	300	500	1,000	SEM	Combined	Linear	Quadratic
78 wk								
CLU	1.16	2.06	1.28	2.22	0.172	0.056	0.105	0.949
OC-116	1.02[c]	1.35[bc]	1.93[ab]	2.30[a]	0.159	0.014	0.001	0.947
OVA	1.15[b]	1.79[ab]	1.71[ab]	2.64[a]	0.178	0.029	0.006	0.655
OVOT	1.18	1.55	0.98	1.13	0.139	0.529	0.566	0.696
83 wk								
CLU	1.18	1.23	1.58	1.98	0.137	0.146	0.028	0.515
OC-116	1.29	2.39	2.55	4.08	0.350	0.054	0.009	0.735
OVA	1.07	1.13	0.47	0.80	0.104	0.095	0.116	0.494
OVOT	1.19	1.75	0.95	1.35	0.140	0.193	0.803	0.769

Abbreviations: CLU, clusterin; LCE, *Lonicera flos* and *Cnicus japonicus* extract; OC-116, ovocleidin-116; OVA, ovalbumin; OVOT, ovotransferrin.
[a-c]Values within a row with different superscripts differ significantly at $P < 0.05$.

Therefore, the effect of LCE addition on egg quality in the results of this study needs further research.

The oviduct of laying hens includes 5 parts: infundibulum, magnum, isthmus, uterus and vagina (Wang et al., 2015). The magnum is the largest part of the fallopian tube and produces the egg-white protein that wraps the yolk (Mishra et al., 2019). The isthmus is responsible for forming 2 layers of eggshell membrane (Arias et al., 1991). When the egg descends to the uterine (or the shell gland), calcite crystal deposition completes eggshell mineralization and eggshell pigment deposition (Gautron et al., 2021), which stays here for about 18 to 22 h and is the longest process of egg formation. In order to explore whether the increase of albumen height, Haugh unit and eggshell thickness caused by LCE supplementation was through the improvement of the health status of the oviduct, the morphology, antioxidant status and immune capacity of the magnum, isthmus and uterine parts of oviduct in laying hens were examined in current study.

In this study, the addition of LCE did not significantly improve the histologic morphology of the uterus in the isthmus of the expanded part, which may be due to the fact that the laying hens were not very old and were not challenged by bacteria, so LCE could not improve the fallopian tubes to a better state. However, it was sufficient to show that the addition of LCE had no harmful effect on the morphology of the fallopian tubes.

Oxidative damage or inflammation of the oviduct caused by disease or environmental factors is a major cause of poor egg quality (Nii, 2022). In the present study, supplementation with LCE significantly decreased the H_2O_2 content in oviduct and MDA content in uterus and increased the CAT and GSH-Px activities in isthmus and T-SOD activity in uterus when compared to the control group. These results indicated that dietary LCE addition could improve the antioxidant status in the oviduct of laying hens. Studies have shown that supplemental CGA could decrease H_2O_2 and MDA content and increase the activities of CAT, T-SOD and GSH-Px (Zhang et al., 2020; Wang et al., 2021) in different animals, which are similar with our results in laying hens. Furthermore, milk thistle extract has a greater effect on increasing CAT and GSH-Px levels because it contains several other flavonoid structures (i.e., apigenin, chrisoeriol, eriodictyol, naringenin, quercetin, taxifolin) (Vessal et al., 2010). However, it needs to be pointed out that over-supplementation of LCE may not enlarge even reverse the beneficial effects by causing unpredictable oxidative metabolic disorder.

The mucosa of oviduct of hens are susceptible to pathogens generally causing inflammation of mucosa, resulting in a deterioration of the health of the host animal, reduced egg production and bacterial contamination of eggs (Nii, 2022). Therefore, the detection of immune-related cytokine expression in the oviduct can evaluate the health status of the oviduct more comprehensively. In present study, LCE supplementation decreased the mRNA expressions of IL-1β in magnum, IFN-γ in magnum and isthmus, TNF-α in magnum and uterus and iNOS in isthmus. The results were in agreement with Kao et al. (2015) who found that pretreatment with *Lonicerae japonicae flos* inhibits inflammatory factors, decreases the mRNA expression of IL-1β, IL-6, and TNF-α in the lung and protects against lung inflammatory cytokine release by LPS induction. In addition, a mixture of aloe vera and milk thistle attenuated the increase in mRNA expressions of TNF-α, iNOS and cyclooxygenase-2 in acute hepatotoxicity (Kim et al., 2009). Furthermore, the mRNA expression of IL-6 in uterus and iNOS in magnum, isthmus and uterus significantly increased in LCE supplementation groups. The expressions of pro-inflammatory and anti-inflammatory must keep in a balance in order to maintain the mucosal homeostasis (Maillard and Snapper, 2010). Study has shown that the expression of pro-inflammatory cytokines IL-1β, IL-6 and IFN-γ is higher in the main stage of eggshell formation (Elhamouly et al., 2018), which was consistent with our experiment. It is more generally believed that anti-inflammatory factors prevent host from being invaded by pathogens (Jiang et al., 2021). Elhamouly et al. (2018) found that a higher expression of the anti-inflammatory cytokines observed in aged hens might disrupt the balance of inflammatory cytokines. Therefore, dietary LCE supplementation may

regulate the well-controlled physiological inflammation occurring in the uterus during eggshell formation.

Based on the results of increased yolk color, albumen height, Haugh unit, and shell thickness causing by LCE supplementation, it was hypothesized that the addition of LCE to the diet might improve the function of oviduct partly by increasing the antioxidant status and immune capacity in oviduct. To test this hypothesis, the mRNA expression levels of shell matrix protein in uterine part of oviduct in laying hens were measured. The avian eggshell primarily consists of calcium carbonate mineral (calcite) and matrix proteins (Li et al., 2019), and the organic matrix of the eggshell could play a role in determining shell quality (Hincke et al., 2010). OC-116 is a main component of extracellular phosphoglycoprotein in eggshell matrix, which is abundant in uterine fluid during calcification and is therefore believed to play a role in the mineralization of eggshell (Hincke et al., 1999; Mikšík et al., 2007; Marie et al., 2015). Gene association analysis revealed that OVA SNPs were associated with eggshell quality measurements of breaking strength and shell thickness (Dunn et al., 2009). In the present study, it was observed that dietary supplementation with LCE significantly increased the mRNA expression of OVA, OC-116 at wk 78, which indicated that LCE had a beneficial effect on eggshell calcification at the late laying period of laying hens. In this study, only the activities of antioxidant enzymes and the expression of some inflammatory cytokines were used to reflect the antioxidant status and immune response in the fallopian tube of laying hens. Therefore, the regulatory mechanism of LCE on the fallopian tube function needs to be further studied.

In conclusion, LCE improved egg quality partly by modulating the activities of antioxidant enzymes and the expression of inflammatory-related cytokines and shell matrix protein in oviduct of laying hens. Further investigation should be conducted to study the role of plant extracts in oxidative stress and immune response of oviduct and the relationship among plant extracts, oviduct health and egg quality.

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DISCLOSURES

The authors declare no conflicts of interest.

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