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Laying Diet Supplementation with *Ricinus communis* L. leaves and Evaluation of Productive Performance and Potential Modulation of Antioxidative Status

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This study evaluated the antioxidant capacity of *Ricinus communis* L. (RC) leaves and powder when used as a feed additive for laying hens. Results showed that the total phenolic content of the aqueous leaf extract of *Ricinus communis* L. (RCE) was 48.39 mg gallic acid equivalent (GAE) per gram dry weight (DW). The flavonoid content was 9.76 mg quercetin dihydrate equivalent (QE)/g DW. Ferrous chelating activity was approximately 56.2% with an RCE concentration of 1 mg/mL; the highest chelating activity was 91.2% with 4 mg/mL extract. The reducing power of 1 mg/mL RC was 1.17 times better than 1 mg/mL butylated hydroxytoluene (BHT). The Trolox equivalent antioxidant capacity (TEAC) value of 12.5 mg/mL RCE was equivalent to 3.09 mg/mL Trolox. RCE (10 mg/mL) had a lipid oxidative inhibition capacity of 35.3%. A total of 80 ISA brown laying hens at twenty-nine weeks of age were randomly allocated into the control or 1 of 3 treatment groups; the latter received 0.5%, 1% or 2% of RC, respectively, for 12 weeks. Results showed that the RC supplementation improved the feed conversion rate and 0.5% RC generated the best results. Additionally, the egg yolk score was significantly increased in all RC-supplemented groups. Moreover, there was no significant difference in serum characteristics between the treatment groups. Serum antioxidant enzyme activity showed that superoxide dismutase (SOD) activity increased in the RC-supplemented groups relative to the control but was not significantly different. mRNA expression levels of the antioxidant regulatory genes *GCLC*, *GST*, *HO-1*, *SOD1*, and *SOD2* were significantly increased with 2% RC supplementation. In summary, RC is a suitable feed additive for laying hens and the addition of 0.5% RC leaf powder resulted in the greatest benefits.

Key words: Antioxidant properties, egg quality, laying hens, *Ricinus communis* L. leaves

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Introduction

In recent years, the trend in feed additives has shifted from antibiotics to probiotics and phytogenics (Lee *et al*., 2017; Teng *et al*., 2017; Lee *et al*., 2019). Unlike the bacterial resistance and environmental pollution caused by antibiotic abuse, phytogenics are a win-win situation for both the environment and pastures as they are natural compounds and are favored by consumers (Abou-Elkhair *et al*., 2018). Phytogenics often have antioxidant, anti-inflammatory, and antibacterial properties (Lee *et al*., 2013). Stressors, including the environment, nutrition, microorganisms and feeding management, have a negative impact on poultry health and production. Oxidative stress (OS) is caused by free radicals generated under pressure that exceed the load of endogenous antioxidant mechanisms (Mishra and Jha, 2019). Free radicals such as superoxides, hydrogen peroxide, and hydroxyl radicals produced by reactive oxygen species (ROS) cause oxidative damage, apoptosis, and DNA damage through cellular signal transduction mechanisms (Liu *et al*., 2008). Previous research found that some substances contained in plants can scavenge free radicals, including flavonoids (e.g., flavonols, flavones, isoflavones, and anthocyanidins), stilbenes, lignans, and phenolic acids. This is not due to a single mechanism but rather, several mechanisms working together (Lee *et al*., 2017). Phenolic compounds in plants have been

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reported to prevent the formation of ROS through non-enzymatic antioxidant pathways (Waśkiewicz *et al*., 2014). Nuclear factor erythroid 2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) coordinate antioxidant responses in animals. Nrf2 is activated by ROS stimulation and binds to the antioxidant response element to initiate phase II enzyme expression [e.g., heme oxygenase-1 (HO-1), glutathione S-transferase (GST), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and glutamate-cysteine ligase catalytic subunit (GCLC)] to enhance the antioxidant system of the cells (Feng *et al*., 2010). Studies have shown that Nrf2 can be activated by many phytogenics, such as herbs, fruits, and plants. Therefore, Nrf2 plays an important role in regulating the enzyme antioxidant system and reducing cell damage caused by ROS (Chen *et al*., 2012; Lee *et al*., 2019).

Ricinus communis L. (RC) is a species of perennial flowering plant in the spurge family *Euphorbiaceae* and is widely found in tropical and subtropical regions. The leaves are often used to feed eri silkworms and the stalks are utilized for fuel in India (Jena and Gupta, 2012). Tribal communities in India use RC as an herb to treat liver disease and research has revealed that RC can protect the liver and prevent lipid oxidation (Babu *et al*., 2017). One of its favored benefits is its use to stabilize sand dunes (Jena and Gupta, 2012). In addition, the economic value of RC mainly comes from its seeds, which are converted to an industrial oil and are currently under research as an alternative energy source and biofuel (Wang *et al*., 2019).

Previous studies showed there are phenolic compounds in dried RC leaves which have strong antioxidant, anti-inflammatory, and antimicrobial activities (Singh *et al*., 2009; Jena and Gupta, 2012). However, limited research and discussion exists on the effects of RC leaf supplementation in animal feed. Thus, the purpose of this paper was to evaluate the influence of RC leaf supplementation on production performance, egg quality, and antioxidant status of laying hens.

Materials and Methods

Plant Collection and Preparation of Extract

RC leaves were obtained from the Miaoli District Agricultural Research and Extension Station of the Council of Agriculture, Executive Yuan. The leaves were dried at 50℃ for 3 h, and then crushed into a fine powder (particle size approximately 1 mm) before being added to the feed. Additionally, RC powder was weighed and added to distilled water, which was extracted at 95℃ for 2 h. The solution was then filtered (Advantec No. 1, Tokyo, Japan) to obtain the RC extract (RCE), which was stored at −20℃ for further analysis.

Quantification of Bioactive Compounds

The total phenolic content was determined using a Folin-Ciocalteu reagent, according to the methods described by Kujala *et al*. (2000). Briefly, 0.5 mL of 1 N Folin–Ciocalteu reagent was mixed with 0.05 mL RCE and 1 mL 7.5% sodium carbonate and allowed to react for 30 min at room temperature (RT, between 25 to 30℃). By comparison with a gallic acid (GA) standard, an equation was obtained from the standard curve of GA and used to determine the phenolic compounds in the RCE (milligram of the GA equivalent, mg GAE). The flavonoid content of the RCE was determined through the colorimetric method (Pourmorad *et al*., 2006). Briefly, 0.5 mL of the RCE was mixed with methanol, 10% aluminum chloride ($AlCl_3$), and 1 M potassium acetate and left at RT for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer and a calibration curve of quercetin solution. The flavonoid content is presented as micrograms of quercetin equivalent (QE) (Table 3).

Ferrous Chelating Capacity Assay

The ferrous chelating capacity was determined according to the methods of Dinis *et al*. (1994). Briefly, 0.25 mL RCE was mixed with 0.025 mL 2 mM ferrous chloride solution and 0.925 mL methanol. After 30 seconds incubation at RT, 0.05 mL 5 mM ferrozine was added to initiate the reaction, and the mixture was left to stand at RT for 10 min. Once the reaction was completed, the absorbance of the mixture was measured spectrophotometrically at 562 nm. The percentage of inhibition of the ferrozine-Fe^{2+} complex was calculated according to the following formula:

Ferrous ion chelating (%)＝[1－(A1/A0)]×100

where A0 and A1 represent the respective absorbance of the control and sample (EDTA and RCE, respectively). The blank was determined by replacing the sample with methanol. In this experiment, EDTA was used as a positive control as it contained ferrous chloride, ferrozine, and complex formation molecules.

Determination of Reducing Power

The reducing power of RCE was measured using the methods described by Oyaizu (1986). RCE was mixed with phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%). The mixture was incubated at 50℃ for 20 min, before the reaction was terminated by adding 2.5 mL 10% trichloroacetic acid. Five milliliters of the upper layer of the solution was then mixed with the same volume of distilled water and 0.1% ferric trichloride. After 10 min of incubation, the absorbance was measured at 700 nm using a spectrophotometer and butylated hydroxytoluene (BHT) as the control. Increased reaction mixture absorbance indicated greater reducing power.

1,1-diphenyl-2-picrylhydrazyl (DPPH) Free Radical Scavenging Capacity Assay

The free radical scavenging ability of the RCE was investigated according to the method of Blois (1958). Briefly, DPPH ethanol solution (1 mM) was prepared and added to the RCE at different concentrations (0−1.0 mg/mL). After 30 min of incubation, the absorbance was measured at 517 nm with a spectrophotometer. Decreased absorbance of the reaction mixture indicated higher scavenging ability. The percentage of DPPH radical scavenging was obtained from the following equation:

DPPH scavenging effect (%)＝100－[1－(A1/A0)×100]

where A0 and A1 represent the respective absorbance of the positive control and sample (BHT and RCE, respec-

tively). The blank was determined by replacing the sample with methanol.

Trolox Equivalent Antioxidant Capacity

Trolox equivalent antioxidant capacity (TEAC) was determined according to the methods described by Gyamfi *et al*. (1999). The TEAC reagent was prepared by mixing 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (1000 μM), H_2O_2 (500 μM), peroxidase (44 U/mL) and distilled water at a ratio of 1:1:1:6, before the solution was left at RT in the dark for 1 h. Diluted RCE (0.1 mL) was then mixed with 0.9 mL TEAC reagent. The reaction mixture was left to stand at RT in the dark for 10 min before the absorbance was measured at 734 nm with a spectrophotometer. The TEAC of the antioxidant was calculated by relating the drop in absorbance to that of a Trolox solution. The results were calculated and plotted with respect to the concentrations of BHT, ascorbic acid, and RCE and expressed in mg/mL of Trolox antioxidant equivalent per gram.

Inhibition of Liposome Oxidation

The inhibitory effect of the RCE on liposome oxidation was tested using the methods described by Yen *et al*. (2002). Briefly, 300 mg lecithin was mixed with 30 mL phosphate buffer (pH 7.4) and the solution was sonicated in an ultrasonic cleaner until it was completely emulsified. Subsequently, the emulsified solution (2 mL, 10 mg lecithin/mL) was mixed with 25 mM ferric trichloride (0.1 mL), 25 mM ascorbic acid (0.1 mL), and RCE at different concentrations. The mixture was incubated for 2 h at 37℃, and the oxidation level was measured using the thiobarbituric acid (TBA) method described by Tamura *et al*. (1991). The absorbance was measured at 532 nm against BHT as a positive control. The inhibition rate was calculated using the following equation:

Inhibition %＝[(A0－A1)/A1]×100

where A0 was the absorbance of the positive control reaction (BHT solution) and A1 was the absorbance in the presence of RCE.

Animals and Experimental Design

A total of eighty 29-week-old ISA brown laying hens were randomly assigned to one of four treatment groups for 12 weeks. Their feed was supplemented with 0% (control), 0.5%, 1%, or 2% RC. Each treatment group involved 10 replicates of 2 birds in individual wire cages (43×40×60 cm). The temperature was set at 25℃ and the relative humidity at 55%. Table 1 shows the ingredients and nutritional content of the basal diet. The diets were formulated to meet or exceed the nutrient requirements of laying hens (National Council, 1994). The proximate composition was analyzed according to the Association of Official Analytical Chemists (1980). All experimental protocols were approved by the Animal Care and Use Committee of National Chung Hsing University (IACUC NO: 106-014). Feed and water were provided *ad libitum* with a light regimen of 17 h of continuous light per day. Eggs were collected, and the egg-laying rate and average egg weight were recorded daily. Feed consumption was recorded on a replicate basis at weekly intervals and calculated as gram/day/bird. The feed conversion rate (FCR) was calculated on a weekly basis for each treatment and expressed as kilograms of feed consumed per kilogram of eggs produced. Eight eggs were collected randomly from each replicate every seven days to measure egg quality during the experimental period. Eggs were weighed and eggshell strength was measured using an eggshell strength tester (DET 6000, NABEL Co., Ltd., Kyoto, Japan). A constantly increasing load was applied to an egg lying lengthways, and eggshell strength was determined based on the time of breakage. Haugh unit values, a measure of egg protein quality, were calculated using egg weight and albumen height, determined using an albumen height analyzer (DET 6000, NABEL Co., Ltd., Kyoto, Japan). Eggshell thickness was determined as the mean of measurements at three locations on the egg (sharp end, blunt end, and middle section) using a dial pipe gauge (Ozaki MFG Co., Ltd., Tokyo, Japan).

Table 1. **Ingredients and nutrient composition of the basal diet**

Ingredients	%
Corn	57.00
Soybean meal (44% of CP)	21.60
Full-fat soybean meal (65% of CP)	1.20
Fish meal	3.00
Corn dried distillers grains with solubles (DDGS)	4.70
Soybean oil	2.00
Acid calcium phosphate	2.60
Calcium carbonate	7.30
Salt	0.15
DL-Methionine	0.13
Choline chloride	0.08
Vitamin-premix[2]	0.125
Mineral- premix	0.125
Total	100
Chemical-nutritional analysis	
ME, kcal/kg	2810
Crude protein, %	17.70
Ether extract, %	5.70
Lysine, %	0.79
Methionine, %	0.41
Methionine + cystine, %	0.71
Calcium, %	3.65
Phosphorus-nonphytate, %	0.50
Phosphorus, %	0.86

[1] The control group was fed the basal diet (corn–soybean meal). The other groups were fed the basal diet supplemented with 0.5%, 1%, or 2% dry *Ricinus communis* L. leaf powder. CP: Crude protein; ME: Metabolizable Energy.

[2] Diet composition per kilogram of feed: vitamin A, 8,000 IU; vitamin D, 1,500 IU; riboflavin, 4 mg; cobalamin, 10 μg; vitamin E, 15 mg; vitamin K, 2 mg; choline, 500 mg; niacin, 25 mg; manganese, 60 mg; zinc, 50 mg; iron, 50 mg; copper, 3 mg; selenium, 0.26 mg.

Determination of Serum Biochemistry

At week 12, 4 birds per treatment group were randomly selected at 8 a.m. for sampling. Blood samples (5 mL) from

a wing-vein puncture were collected into nonheparinized tubes, incubated at 37℃ for 2 h, and then centrifuged at 3,000×*g* for 10 min. Serum biochemistry, including glucose, blood urea nitrogen (BUN), creatinine, uric acid, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), total protein, albumin, globulin, alkaline phosphatase, triglycerides, high-density lipoproteins, low-density lipoproteins, and total cholesterol concentration, were examined by the Health-Medical Laboratory, Taiwan.

Determination of Serum Antioxidant Enzymes Activities

Superoxide dismutase (SOD) and catalase (CAT) activity were measured calorimetrically using a spectrophotometer. The procedures were conducted using an assay kit purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Briefly, SOD activity was detected by adding 200 μL radical detector and 10 μL serum to each well of a 96-well plate, before the reaction was started by adding 20 μL xanthine oxidase. After incubation on a shaker for 30 min at RT, the absorbance at 440 nm was measured using a plate reader. SOD activity was calculated using the manual provided with the assay kit. One unit of SOD activity was defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical. A CAT assay was performed by adding 100 μL diluted potassium phosphate buffer (100 mM, pH 7.0), 30 μL methanol, and 20 μL serum to each well of a 96-well plate. The reaction was initiated by adding 20 μL hydrogen peroxide (0.03 M) to all wells, followed by incubation on a shaker for 20 min at RT. The reaction was terminated by adding 30 μL CAT Purpald. After 10 min of incubation on a shaker at RT, 10 μL CAT potassium periodate was added and the samples were incubated a further 5 min. Once the reaction was complete, the absorbance of the mixture was measured spectrophotometrically at 540 nm using a plate reader. Formaldehyde content was obtained using the standard curve and CAT activity was calculated using the manual provided with the assay kit. One unit of CAT was defined as the amount of enzyme that would cause the formation of 1.0 nmol formaldehyde per minute at 25℃. The entire reagent was provided and adjusted to the finest concentration using the assay kit. Antioxidant enzyme activity was expressed as units (U) per milliliter of serum.

Peripheral Blood Mononuclear Cell Collection

Collected blood was layered on 1077 Histopaque (Sigma, 10771) and centrifuged at 200×*g* for 10 min. Peripheral blood mononuclear cells (PBMCs) were collected from the gradient interface. The plasma suspension was combined and washed three times with phosphate buffered saline and then centrifuged at 200×*g* for 10 min. After the suspension was removed, 1 mL RNAzol reagent (Sigma-Aldrich, St. Louis, MO, USA) was added, and the result was stored at −80℃.

mRNA Concentration and RNA Conversion to cDNA of Antioxidant Related Genes

Total RNA was isolated from cultured PBMCs using the RNAzol reagent, according to the manufacturer's protocol for the determination of mRNA expression. Determination of total RNA concentration, purity, cDNA synthesis, and qPCR analysis was performed and modified as per the methods of Lin *et al*. (2014). Gene-specific primers were designed according to the genes of *Gallus gallus* (chickens); Table 2 lists the features of the primer pairs. After normalizing the gene expression data with the calculated GeNorm normalization factor, the means and standard devia-

Table 2. **Primers used for qPCR analysis and characteristics of the PCR products**

Gene	Forward primer (from 5′ to 3′) Reverse primer (from 5′ to 3′)	PCR product size (bp)	NCBI GenBank
β-actin	CTGGCACCTAGCACAATGAA ACATCTGCTGGAAGGTGGAC	109	X00182.1
HO-1[1]	AGCTTCGCACAAGGAGTGTT GGAGAGGTGGTCAGCATGTC	106	X56201.1
GST[2]	AGTCGAAGCCTGATGCACTT TCTAGGCGTGGTTTCCTTTG	121	L15386.1
Nrf2[3]	GGAAGAAGGTGCTTTTCGGAGC GGGCAAGGCAGATCTCTTCCAA	171	NM_205117.1
GCLC[4]	CAGCACCCAGACTACAAGCA CTACCCCCAACAGTTCTGGA	118	XM_419910.3
SOD1[5]	ATTACCGGCTTGTCTGATGG CCTCCCTTTGCAGTCACATT	173	NM_205064.155
SOD2[6]	GCCACCTACGTGAACAACCT AGTCACGTTTGATGGCTTCC	208	NM_204211.1

[1] HO-1 - heme oxygenase -1.
[2] GST - glutathione S-transferase.
[3] Nrf2 - nuclear factor (erythroid-derived 2)-like 2.
[4] GCLC - glutamate-cysteine ligase, catalytic subunit.
[5] SOD1 - superoxide dismutase 1.
[6] SOD2 - superoxide dismutase 2.

tions (SDs) were calculated from the normalized expression data for samples from the same treatment groups.

Statistical Analyses

Data was subjected to ANOVA as a completely randomized design using the GLM procedure of the SAS software (SAS, 2004). Significant statistical differences among the mean values of the four treatment groups were determined using Tukey's honestly significant difference test with a significance level of $P<0.05$.

Results

Chemical Composition and Bioactive Compounds in RC

Table 3 shows the chemical composition of RC. The dry matter, crude ash, crude protein, acid detergent fiber (ADF), neutral detergent fiber (NDF), and ether extract were determined to be 90%, 9.42%, 20.6%, 16.8%, 25.1%, and 2%, respectively. Table 3 also shows the content of the bioactive compounds of RC. The total phenolic content was 48.39 mg of GAE/g DW, and total flavonoids were 9.76 mg of QE/g DW.

Antioxidant Activity

The ferrous ion-chelating capacity of the RCE is represented in Figure 1A. Compared to 0.1 mg/mL EDTA, the chelating capacity of RCE was 56.24% at a concentration of 2 mg/mL. The increasing chelating capacity of the RCE slowed once the RCE concentration exceeded 2 mg/mL and increased only slightly beyond that. Figure 1B illustrates the reducing power of the RCE. The reducing power of 1 mg/mL RC was 1.17 times better than 1 mg/mL of BHT. The reducing power of RC increased slightly with increased concentration. The free radical scavenging capacity of the RCE is represented in Figure 1C. With 0.25 mg/mL, the scavenging effect of BHT was 87.6%, and the RCE exhibited scavenging effects of 16.8% and 26.7% with 0.125 and 0.25 mg/mL, respectively. Figure 1D represents the TEAC value of the RCE. A TEAC value of 12.5 mg/mL was slightly higher than the TEAC value for ascorbic acid (6.25 mg/mL). The TEAC observed when 25 mg/mL of the RCE was added was approximately equivalent to 1.5625 mg/mL BHT. Figure 1E illustrates the liposome oxidation inhibition ability of the RCE. When the BHT concentration was 2.5 mg/mL, its inhibitory ability was 63.76%, which gradually increased with its concentration. For 10 mg/mL of vitamin C, the inhibitory ability was 58.67%, which gradually increased with concentration. In comparison, RCE concentrations of 10 mg/mL and 20 mg/mL demonstrated inhibitory abilities of 35.29% and 51.06%, respectively. The increase in the RCE's inhibitory ability decreased once the RCE concentration exceeded 20 mg/mL and increased only slightly after that.

Table 3. **Chemical composition and content of the bioactive compounds of *Ricinus communis* L.**

Items	
Chemical composition	
Dry matter (%)	90.2±0.37
Crude ash (%)	9.42±0.36
Crude protein (%)	20.6±0.28
ADF (%)	16.8±0.44
NDF (%)	25.1±0.98
Ether extract (%)	2.0±0.002
Bioactive compounds	
Total phenolics (mg of GAE[2]/g DW[3])	48.39±0.98
Total flavonoids (mg of QE[4]/g DW)	9.76±0.70

[1] Each value represents the mean of three replicates ($n=3$).
[2] GAE: Gallic acid equivalent.
[3] DW: Dry weight.
[4] QE: Quercetin equivalent.

Production Performance

Table 4 shows the effects of dietary RC supplementation on hen performance from weeks 1 to 12. All hens remained healthy and no mortality occurred throughout the study period. There was no significant difference in feed intake, laying rate, or egg weight between the control group and those that received RC; however, the FCR of the hens in the 0.5% RC treatment group was significantly ($P=0.013$) lower than the control.

Egg Quality

Table 5 shows the effects of RC supplementation on egg quality from weeks 1 to 12. There was no significant difference in egg weight, eggshell strength, Haugh unit, or shell thickness between the control group and those that received RC. However, the yolk color scores in all RC-treated groups (0.5% RC, 1% RC, and 2% RC) were significantly ($P<0.0001$) higher than the control.

Serum Biochemistry and Antioxidant Enzyme Activities

Table 6 shows the effects of RC supplementation on the serum biochemistry of the hens at 12 weeks. There was no significant difference in serum biochemistry between all treatment groups and the control. Table 7 shows the effects of RC supplementation on the serum antioxidant enzyme activity in the hens at 12 weeks. Compared to the control, hens that received RC supplementation expressed higher SOD activity ($P<0.1$). However, there was no significant difference in CAT activity among any of the groups.

Antioxidant-regulated Genes in Chicken PBMCs

Figure 2 shows the effects of RC supplementation on the antioxidant-regulated genes of the hens at 12 weeks. The expression levels of *GST*, *GCLC*, *HO-1*, *SOD1*, and *SOD2* were significantly ($P=0.0024$, 0.0367, 0.0044, <0.0001, and <0.0001, respectively) higher in hens supplemented with 2% RC, whereas the 1% RC treatment group demonstrated significantly higher expression of *GCLC*, *HO-1*, *SOD1*, and *SOD2*, compared to the control. Additionally, the 0.5% RC group expressed significantly increased levels of *SOD1* and *SOD2* compared to the control.

Discussion

Studies have suggested that RC leaves contain large amounts of phytochemicals, which provide antioxidant, antibacterial, and anti-inflammatory abilities (Oyewole *et al*., 2010). Jena and Gupta (2012) found that the main bioactive compounds in RC leaves are present in phenolic and flavonoid compounds; similar results were reported by Singh *et*

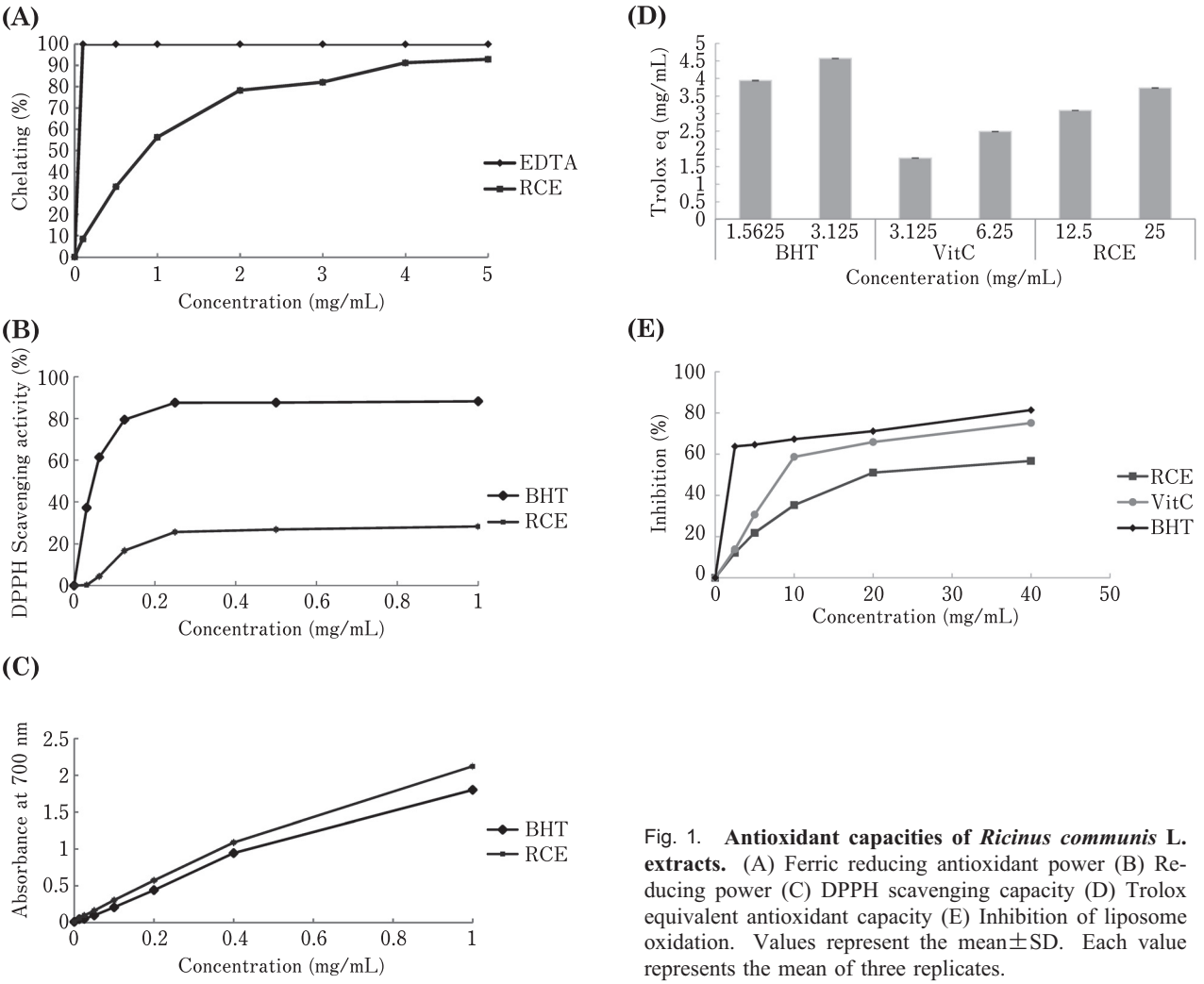

Fig. 1. **Antioxidant capacities of *Ricinus communis* L. extracts.** (A) Ferric reducing antioxidant power (B) Reducing power (C) DPPH scavenging capacity (D) Trolox equivalent antioxidant capacity (E) Inhibition of liposome oxidation. Values represent the mean±SD. Each value represents the mean of three replicates.

al. (2009). The results of our study showed that the total phenolic and flavonoid content of RCE was 48.39 mg GAE/g and 9.76 mg QE/g, respectively (Table 3). The antioxidant activities of phenolics and flavonoids are mainly derived from aromatic rings with one or more hydroxyl moieties (Lee *et al*., 2017), which provide hydrogen ions and chelate free radicals to exhibit antioxidant activity (Amri and Hossain, 2018). Flavonoids can also inhibit the production of ROS (Bakoyiannis *et al*., 2019).

The chemical antioxidant capacity of plants cannot be determined by a single evaluation method since it is achieved via the interaction of multiple compounds (Atere *et al*., 2018). Therefore, this study used five methods for measuring antioxidant capacity to demonstrate the antioxidant capacity of the RCE. Fe^{2+} produced in animals causes the generation of hydroxyl radicals, leading to lipid oxidation. Chvátalová *et al*., (2008) reported that polyphenols in plants can chelate transition metal ions. RCE could also chelate ferrous ion and reduce peroxidation of ferrous thereby inhibit hydroxyl radicals produce and further decrease the liposome oxidation (Figure 1A & 1D). Loganayaki *et al*. (2013) reported that the antioxidant activity in the extract reduced the Fe^{3+}/ferricyanide complex to its ferrous form. As shown in Figure 1B, the RCE donates electrons and hydrogen atoms to break free radical chains and prevent the formation of peroxide (Loganayaki *et al*., 2013). Antioxidants provide the hydrogen ions needed to transform DPPH radicals into DPPH-H; therefore, DPPH radicals can be used to evaluate the ability of natural compounds to provide hydrogen ions (Singh *et al*., 2009). TEAC assays are widely used to evaluate the total amount of free radicals that can be scavenged by antioxidants (Arts *et al*., 2004). However, based on the results of these two analyses, RCE did not demonstrate good scavenging ability. Natural phenolic compounds have a wide variety of molecular structures, so they have distinct free radical scavenging activities due to hydroxylation, glycosylation, and methoxylation (Cai *et al*., 2006). In the current study, the antioxidant properties, including ferric-reducing antioxidant power (Figure 1A), reducing power (Figure 1B), and inhibition of liposome oxidation (Figure 1E), demonstrated that the RCE contained abundant phenolic and flavonoid components. In addition, it showed that RC

Table 4. **Effects of diet supplementation of *Ricinus communis* L. leaf powder on the production performance of laying hens**

Item	Treatments[1]				SEM[3]	*P*-values
	Control	0.5%	1%	2%		
1−12 weeks						
Feed intake, g/day/bird	105.3	103.2	104.7	104.9	3.14	0.988
Laying rate, %	91.3	93.2	92.3	92.5	1.18	0.718
Egg mass, g/day/bird	57.3	57.4	57.4	57.2	0.24	0.942
FCR[2], %	1.92[a]	1.79[b]	1.86[a]	1.89[a]	0.02	0.013

[1] Control: basal diet; 0.5%: 0.5% *Ricinus communis* L. leaves; 1%: 1% *Ricinus communis* L. leaves; 2%: 2% *Ricinus communis* L. leaves. Results are provided as the mean of eight replicates in each control and treatment group ($n=8$).
[2] FCR: feed conversion rate.
[3] SEM: standard error of the mean.
[a, b] Means within the same rows without the same superscript letter are significantly different ($P<0.05$).

Table 5. **Effects of diet supplementation of *Ricinus communis* L. leaf powder on the egg quality of laying hens**

Item	Treatments[1]				SEM[2]	*P*-values
	Control	0.5%	1%	2%		
1−12 weeks						
Egg weight, g	57.3	57.4	57.4	57.2	0.24	0.941
Eggshell strength, kg/cm^2	4.10	4.08	4.19	4.07	0.05	0.421
Haugh unit, HU	81.2	81.9	83.0	81.5	0.77	0.404
Yolk color score	3.8[c]	4.8[b]	5.0[ab]	5.2[a]	0.13	<0.0001
Shell thickness, mm	0.36	0.36	0.37	0.36	0.01	0.840

[1] Control: basal diet; 0.5%: 0.5% *Ricinus communis* L. leaves; 1%: 1% *Ricinus communis* L. leaves; 2%: 2% *Ricinus communis* L. leaves. Results are provided as the mean of eight replicates in each control and treatment group ($n=8$).
[2] SEM: standard error of the mean.
[a, b, c] Means within the same rows without the same superscript letter are significantly different ($P<0.05$).

had a high antioxidant capacity.

The benefits of dietary supplementation in poultry with phytogenics have previously been investigated (Lee *et al*., 2017). Lin *et al*. (2017) reported that dietary supplementation with mulberry leaves improves the performance of laying hens since their antioxidant status improved. Our results showed that 0.5% supplementation with RC improved the FCR ($P<0.05$), which could also be explained by the increased antioxidants in the hens. Our results are similar to those of Amirshekari *et al*. (2016), who reported that an increase in antioxidants does not necessarily lead to a better production performance. This was verified by Lee *et al*. (2013) who found that dietary supplementation with 0.5% *Echinacea purpurea* L., versus higher concentrations, generates the best FCR in broilers.

Our results showed that dietary supplementation with RC powder had no effect on egg weight, eggshell strength, Haugh unit, or shell thickness. However, egg yolk color was significantly increased. This result is similar to Cayan and Erener (2015), who reported that supplementation with olive leaf (*Olea europaea*) powder has no significant effect on egg weight in laying hens but significantly increases egg yolk color. Similar results were obtained by Ghasemi *et al*. (2010), who found that the addition of garlic and thyme does not affect the Haugh unit or eggshell thickness but increases egg yolk color and egg weight. We speculated that the yolk color in the RC-supplemented group was due to the carotenoid pigment in RC. The depth of pigmentation of the egg yolk reflected the level of carotenoids in the feed. Consumers in most countries prefer eggs with a deeply colored yolk, which increases consumer acceptance and increases profits (Grashorn 2016). However, the result of phytogenic supplementation on egg quality is influenced by the different types of phytochemicals and active materials available, which are dependent on the parts of the plant, geographic location, harvesting season, etc. Moreover, egg quality is also affected by the variety of laying hens and the feeding environment (Alloui *et al*., 2014).

Babu *et al*. (2017) reported that RC leaf extracts exhibit hepatoprotective properties in albino rats and indicate that bioactive compounds are their primary source of efficacy. Our results are similar to Toghyani *et al*. (2010), who reported that broilers supplemented with black seeds (*Nigella sativa*) and peppermint (*Mentha piperita*) show no significant differences in the concentrations of serum protein, albumin, triglyceride, LDL, HDL and total cholesterol, SGOT or SGPT. In addition, there was no decrease in laying rate or mortality of the hens. Therefore, an appropriate amount of

Table 6. **Effects of diet supplementation of *Ricinus communis* L. leaf powder on the serum biochemistries of laying hens**

Item	Treatments[1]				SEM[2]	*P*-values
	Control	0.5%	1%	2%		
Glucose, mg/dL	197.5	214.0	222.8	221.7	8.57	0.209
BUN, mg/dL	4.3	4.8	3.3	3.5	1.01	0.714
Creatinine, mg/dL	0.1	0.1	0.1	0.1	0.03	0.390
Uric acid, mg/dL	4.0	3.7	4.2	3.2	0.42	0.488
SGOT, U/L	155.5	151.0	143.0	142.5	9.11	0.699
SGPT, U/L	1.0	1.3	1.3	1.0	0.18	0.452
Total protein, g/dL	4.7	4.2	4.8	4.6	0.18	0.188
Albumin, g/dL	1.9	1.7	1.9	1.9	0.05	0.124
Globulin, g/dL	2.9	2.5	2.9	2.7	0.15	0.272
Alkaline phosphatase, IU/L	632.8	571.5	517.8	787.5	107.76	0.361
Triglycerides, mg/dL	1517.5	1721.3	1947.3	1742.3	272.40	0.745
HDL cholesterol, mg/dL	43.3	44.5	46.5	44.0	4.10	0.950
LDL cholesterol, mg/dL	21.3	24.5	31.8	27.3	4.76	0.482
Total cholesterol, mg/dL	90.8	93.5	103.3	94.3	5.06	0.370

[1] Control: basal diet; 0.5%: 0.5% *Ricinus communis* L. leaves; 1%: 1% *Ricinus communis* L. leaves; 2%: 2% *Ricinus communis* L. leaves. Results are provided as the mean of four replicates in each control and treatment group (n=4).
BUN=blood urea nitrogen; SGOT=serum glutamic oxaloacetic transaminase; SGPT=serum glutamic-pyruvic transaminase.
[2] SEM: standard error of the mean.

Table 7. **Effects of diet supplementation of *Ricinus communis* L. leaf powder on the serum antioxidant enzyme activities of laying hens**

Item[3]	Treatments[1]				SEM[2]	*P*-values
	Control	0.5%	1%	2%		
SOD (U/mL)	37.59$^{(b)}$	42.72$^{(a)}$	42.98$^{(a)}$	43.54$^{(a)}$	1.65	0.069
CAT (nmol/min/mL)	51.23	53.73	67.41	65.14	9.15	0.524

[1] Control: basal diet; 0.5%: 0.5% *Ricinus communis* L. leaves; 1%: 1% *Ricinus communis* L. leaves; 2%: 2% *Ricinus communis* L. leaves. Results are provided as the mean of six replicates in each control and treatment group (n=6).
[2] SEM: standard error of the mean.
[3] CAT and SOD represent catalase, superoxide dismutase, respectively.
[(a),(b)] Means within the same rows without the same superscript letter show a trend (a, b in parentheses; $P<0.1$).

RC powder supplementation did not adversely affect laying hens. Cells develop nonenzymatic and enzymatic antioxidant systems to limit damage from free radicals and ROS. Enzymatic antioxidants are endogenously synthesized and regulated and are important indicators of the oxidation state of animal tissues (Lin *et al*., 2017). SOD and CAT are two endogenous antioxidant enzymes that constitute the antioxidant cellular enzymatic system, and act as a first line of defense. SOD catalyzes the dismutation of a superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and an oxygen molecule (O_2) to reduce the damage caused by a superoxide anion. SOD also works in conjunction with CAT, which converts H_2O_2 to H_2O (Ighodaro and Akinloye 2018). Lee *et al*. (2015) reported that dietary supplementation with *Pleurotus eryngii* stalk residue (PESR) improves the serum antioxidant status as measured by CAT and SOD values, which may be an effect of the flavonoid compounds in PESR. The increased SOD activity in the RC-supplemented feed in our study indicated that phenolic compounds in RC played a role in increasing the level of antioxidant enzymes and enhancing the ability of laying hens to scavenge free radicals and ROS.

Nrf2 binds to negative regulator protein keap1 and is sequestered in the cytoplasm and inhibited under basal conditions. Nrf2 is activated in the nucleus when cells suffer oxidative stress (Pandey *et al*., 2017). The binding of activated Nrf2 to the antioxidant response element (ARE) initiates antioxidant gene transcription and synthesis of phase II enzymes such as GCLC, GST, HO-1, SOD1, and SOD2. Phase II enzymes protect cells against oxidative stress and thus constitute a protective antioxidant defense system (Hur *et al*., 2010). Lee and Johnson (2004) and Liang *et al*. (2013) indicate that phenolic compounds can increase Nrf2 levels to enhance the levels of phase II enzymes GCLC, GCLM, and GST, and increase the GSH production of nonenzymatic antioxidant systems. An increase in HO-1 activity

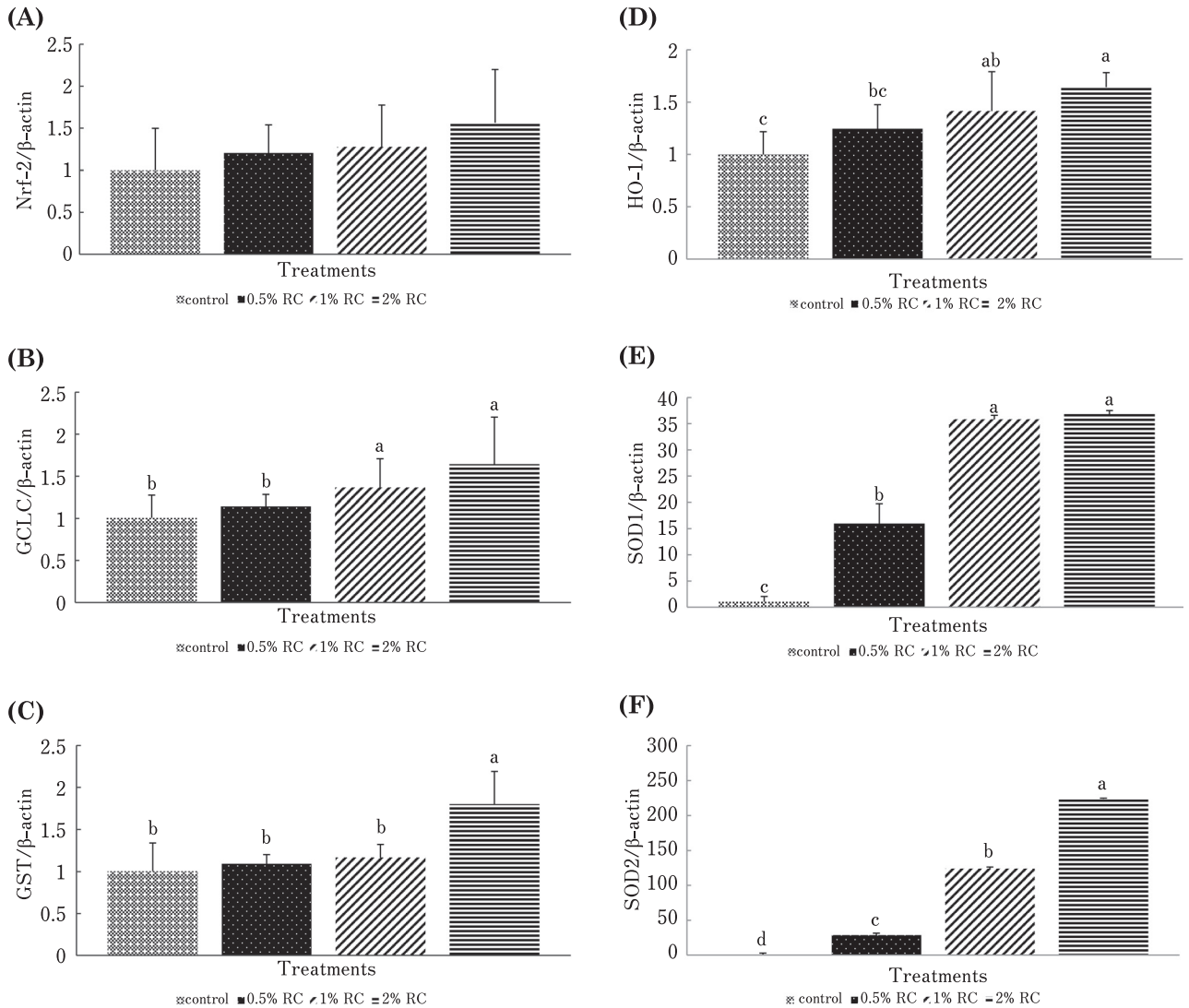

Fig. 2. **Effects of diet supplementation with *Ricinus communis* L. leaf powder on mRNA expression levels of antioxidant-regulated genes of laying hens.** Each value represents the mean of four replicates, and the values are presented as the mean±SD. $^{a, b, c, d}$ Means within the same rows without the same superscript letter are significantly different ($P<0.05$).

allows cells to avoid oxidative damage (Song *et al*., 2018). Phenolic compounds elevate mRNA levels of SOD1 and SOD2 in duck livers (Lin *et al*., 2017). In our study, the mRNA levels of phase II enzymes GCLC, GST, HO-1, SOD1 and SOD2 were significantly increased by dietary supplementation with RC. Lee *et al*. (2017) reported that phenolic components can interfere with multiple cell-signaling pathways and in larger doses they could be used in their natural form to alleviate stress in animals.

In summary, our results showed that dietary supplementation with RC in laying hens improved the FCR, egg yolk color, serum antioxidant enzyme activity, and activated the Nrf2 gene to increase phase II enzyme performance. RC leaves could be used as phytogenic feed additives and supplied to laying hens. In addition, 0.5% supplementation generated the best results in the FCR, while 2% supplementation increased the phase II enzyme performances levels.

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Conflicts of Interest

No potential conflict of interest was reported by the authors.

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