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The regulative effect of *Urtica dioica* on sex hormones imbalance: elevated follicle-stimulating hormone/luteinizing hormone ratio ≥ 4.5 is associated with low performance in aged breeder quails

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ABSTRACT

The age-related reproductive disorders are the main concerns in old birds. It was suggested that a drop in egg production and reproductive performance, towards the end of their laying period was caused partly by a decrease in the baseline concentration of plasma LH. *Urtica dioica* (nettle) is a plant with natural aromatase inhibitors. Steroid hormone levels are regulated by inhibition of the aromatase enzyme. Few studies have examined the effect of nettle on the egg production in adult hens. The aim of this study was to investigate the effects of diet supplemented with nettle powder (NP) in aged quails. One hundred and forty-52-week-old Japanese quails were randomly assigned to four treatments consisting of seven replicates ($n = 5$; four females and one male) and fed with diets containing NP at 0% (control group), 0.5, 1.0, and 1.5% (treatment groups). At 62 week of age, our results indicated the NP improved egg production, feed conversion ratio, eggshell thickness and Haugh unit ($p < .05$). Notably, fertility and hatchability of fertile eggs were significantly increased, while total embryonic mortality decreased significantly by supplementing diet with nettle powder ($p < .05$). Higher luteinizing hormone, lower oestrogen, malondialdehyde and total cholesterol and triglyceride concentrations were associated with percent of the nettle powder in diet ($p \leq .05$). Elevated follicle-stimulating hormone/luteinizing hormone ratio ≥ 4 is associated with low egg production in control group and nettle supplementation can balance FSH/LH ratio to ≤ 2.7 . It is concluded that nettle powder could be used as a worthwhile feed additive at the late laying period of aged quails.

HIGHLIGHTS

- Nettle powder can be used as a food additive with aged quails at late laying period.
- Addition of 1 and 1.5% nettle powder improves egg production, FCR, egg shell thickness.
- Nettle enhances reproductive performances, such as fertility, hatchability, weight of ovary, and weight of follicles by balancing reproductive hormones at late laying period.

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Introduction

The age-related disorders; lower percentage of egg production (Joyner et al. 1987; Burke and Attia 1994), follicular atresia (Holmes and Ottinger 2003; Holmes et al. 2003), lower rate of follicular development (Holmes and Ottinger 2003), decline in clutch size (Grossman et al. 2000), thinner eggshells and a higher percentage of cracked eggs (Roland 1979; Attia et al. 1994) are the main concerns at late laying period. Furthermore, reductions in the number of fertilised eggs and infertility (30–50% by 18 months of age) are the major economic losses in the poultry industry

(Ottinger 2001; Holmes and Ottinger 2003). The process of reproductive senescence in female birds is characterised by a gradual transition from regular reproductive cycles to irregular cycles to eventual acyclicity, and ultimately a low or loss of fertility at the end of the production period. Several studies have confirmed the suppressor effects of oestrogen on FSH and LH levels during the follicular phase in females (Messinis and Templeton 1990; Messinis 2006). It is well-known that low-level oestrogen imbalance define the gonadotropin secretions in the female by reducing hypothalamic releasing of GnRH (Attia et al. 1994; Shaw et al. 2010). There are now significant evidence

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to emphasis that the reproductive hormones undergo significant changes with ageing animals (Wise et al. 1997; Yin and Gore 2006; Shaw et al. 2010). It has been shown that the LH and FSH responses to GnRH were attenuated in older compared with younger females. The oestrogen has an inhibitory effect on FSH releasing was significantly attenuated with ageing (Shaw et al. 2010). As aromatase inhibitors, which decrease the plasma concentration of oestrogens, can be considered to be valuable medicines as a therapeutic method in the treatment of oestrogen dependent disorders. The tendency to use herbs and herbal extracts as healthy food additives has increased over time in poultry nutrition, due to less side effects and chemical additives (Alcicek et al. 2003; Attia et al. 2011; Attia et al. 2017). There are several evidences that confirm the Nettle (*Urtica dioica*) products inhibit the aromatase and interfere with the conversion of testosterone into oestrogens (Kraus et al. 1991; Chrubasik et al. 2007). Thus, aromatase inhibition administration may be a novel means of normalising oestrogen levels and consequently to optimise the follicle-stimulating hormone/luteinizing hormone ratio in the elderly female by increased LH level that result in an improvement in fertility and egg production in birds. To test this hypothesis, and to explore the physiologic effects of aromatase inhibition in aged hens, we administered the nettle powder as a plant-based aromatase inhibitor to influence the egg production in the old layer quails.

Materials and methods

Collection and identification of plant material

Nettle plant used for the investigation was collected from a local area in Kermanshah, Iran. Each part of aerial parts and roots were dried separately under shade at low humidity and 25–32 °C for about 30 days and grounded. Plant identification was confirmed according to the book collection of Iran Herbs (Ghahraman 1984).

Gas chromatography-mass spectroscopic analysis

Two common solvents were used for the extraction of bioactive compounds from the aerial parts and root of NP including water and ethanol. The sample was macerated in these solvents for two days by adding 100 g of sample into 90 mL of solvent and the solution was then filtered using filter paper (Whatman No. 1) and then concentrated in a rotary evaporator at 40 °C and stored at –20 °C for subsequent evaluation.

Table 1. Dietary ingredients, nutrient composition, and metabolisable energy of the experimental diet (% on dry matter basis).

Item	NP0	NP0.5	NP1	NP1.5
Ingredients				
Corn	53.17	53.17	53.17	53.17
Soybean meal	35	35	35	35
Corn oil	3.79	3.79	3.79	3.79
Limestone	5.43	5.43	5.43	5.43
Dicalcium phosphate	1.63	1.63	1.63	1.63
Common salt	0.34	0.34	0.34	0.34
Mineral premix*	0.25	0.25	0.25	0.25
Vitamin premix**	0.25	0.25	0.25	0.25
DL-Methionine	0.14	0.14	0.14	0.14
Stinging nettle powder (SNP)	0	0.50	1	1.50
Calculated analysis				
ME (Kcal/Kg)	2900	2900	2900	2900
Croude protein (%)	20	20	20	20
Calcium (%)	2.50	2.50	2.50	2.50
Available phosphorus (%)	0.40	0.40	0.40	0.40
Sodium (%)	0.15	0.15	0.15	0.15
Arginin (%)	1.26	1.26	1.26	1.26
Lysin (%)	1.07	1.07	1.07	1.07
Methionine + Cysteine (%)	0.77	0.77	0.77	0.77

ME: metabolisable energy.

*Mixture supplied per kg of diet: retinyl acetate, 1.8 mg; cholecalciferol, 0.025 mg; dl-tocopheryl acetate, 1.25 mg; menadione sodium bisulphite, 2.5 mg; thiamine-hydrochloride, 1.5 mg; riboflavin, 3 mg; D-pantothenic acid, 5 mg; pyridoxine hydrochloride, 2.5 mg; vitamin B-12, 0.0075 mg; folic acid, 0.25 mg; niacin, 12.5 mg.

**Mixture supplied per kg of diet: Mn (MnSO₄ H₂O), 50 mg; Fe (FeSO₄ 7H₂O), 30 mg; Zn (ZnO), 30 mg; Cu (CuSO₄ 5H₂O), 5 mg; I (KI), 0.5 mg; Se (Na₂SeO₃), 0.15 mg; Co (CoCl₂ 6H₂O), 0.1 mg; choline chloride, 125 mg.

Quantitative and qualitative data of essential oils were determined by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was done at 290 °C on an Agilent 7890 A gas chromatograph. The GC column was as follows: HP-5MS; the size of fused silica capillary was 0.25 × 3000 × 0.25 µm film thickness and the helium was used as the carrier gas with a flow rate of 0.8 ml/min. The GC column used was programmed as follows: 45 °C (5 min), 280 °C at the rate of 5 °C/min. The injection temperature was 280 °C. The mass spectrometer was operating in E1 mode at 70 eV. The compounds of essential oil were identified tentatively by comparing their relative retention times and mass spectra with those of pure authentic samples. The percentages and the retention indices of bioactive compounds from ethanol and aqueous extracts of different parts of nettle by the GC-MS analysis are listed in Table 1.

Birds, housing, and feeding

One hundred and forty laying breeder Japanese quails (52 weeks old, average weight = 258 g) were randomly assigned to four treatments consisting of seven replicates ($n = 5$; four females and one male) and fed with diets containing NP (%) at 0 (control group), 0.5, 1.0, and 1.5 plant powder is made of the whole its parts

(both root and aerial parts) dried, as the treatment groups (Mansoub 2011). The basal diet (Table 1) was formulated based on the recommendation of NRC . After a 2-week adaptation period, lighting programme (16 h light: 8 h dark), ambient relative humidity (55–60%), and temperature ($22 \pm 1^\circ\text{C}$) was maintained throughout the experimental period.

Production performance parameters

Egg production (daily-recorded), egg weight (daily-recorded), feed intake, and feed conversion ratio were evaluated per replicates.

Egg quality characteristics

To access egg weight, yolk percentage [(yolk weight/egg weight)*100], albumen percentage [(albumen weight/egg weight)*100] and eggshell thickness seven eggs per treatment were measured with a digital balance once each second week (in total, 28 eggs per treatment).

Eggshell thickness was calculated using a dial-gauge micrometre for each egg at three points (top, middle, and bottom) and the average was recorded. Yolk colour scores by DSM dye-colored blades and Haugh unit score (HU) were measured by using formulae as $\log(\text{albumen height} + 7.57 - 1.7 \times \text{Egg weight } 0.37) \times 100$ (Brant 1951).

Reproductive performance parameters

Fourteen eggs per replicate were collected at 2, 4, 6, and 8 weeks of experiment and stored at 15°C before incubation. Altogether, 1568 eggs were incubated for this experiment. Fertility rate [(fertile eggs/total eggs)*100], hatchability of set eggs [(hatch eggs/total eggs)*100], hatchability of fertile eggs [(hatch eggs/fertile eggs)*100] were calculated per replication. Unhatched eggs were analysed for embryonic mortality and were classified as early, middle, and late dead.

Morphological parameter of ovary

To evaluate the ovary's characteristics, on the last day of the experiment, one female quail from each cage was randomly weighed and sacrificed. The ovary and oviduct weight were scaled and calculated as a percentage of the carcase weight. The diameters of follicles were measured by caliper and classified hierarchical follicles ($>10\text{ mm}$), large yellow follicles

(5–10 mm), and their number was calculated (Renema et al. 2001).

Biochemical parameters

Collected blood samples (1.5 mL blood; one sample per replicate and totally seven samples for treatment) through wing vein in tubes that impregnated with EDTA centrifuged at $3000 \times g$ for 10 min and plasma stored at -20°C . Luteinizing hormone (LH), follicle-stimulating hormone (FSH), oestrogen, progesterone, and testosterone concentrations in the plasma were measured by ELISA (optical density at 450 nm) according to the manufacturer's instructions (Monobind Inc., Costa Mesa, CA, USA). Malondialdehyde (MDA) levels, activities of superoxide dismutase (SOD), catalase (CAT) with, and glutathione peroxidase (GPX) were measured in samples using spectrophotometer. Optical density for MDA, SOD, CAT, and GPX was at 532, 560, 240, and 412 nm, respectively. To evaluate plasma levels of triglycerides, total cholesterol, LDL-cholesterol, and HDL-cholesterol, samples were analysed spectrophotometric on the corresponding reagent kit (Pars Azmun, Tehran, Iran).

Statistical analysis

Data were analysed using analysis of variance (ANOVA) following the general linear model procedure of SAS . Treatment means were separated by Tukey's *post-hoc* test. The model used for data analysis was; $Y_{ij} = \mu + T_i + e_{ij}$, where Y_{ij} = the response variable (the observation in i th treatment), μ = the overall mean, T_i = true effect of the i th treatment, and e_{ij} = the random error.

Results

GC-MS analysis of compounds was carried out in ethanolic and aqueous aerial and root extracts of *U. dioica*, and the active principles with their retention time (RT) and concentration (peak area %) are presented in Table 2. Chemical analysis by GC-MS indicated 3,4,5-trihydroxybenzoic acid ethyl ester (25.6%), silane (14.5%), and other benzene derivatives (44%) are the main components in the aqueous extracts of nettle root powder. The major oil component of the aqueous extract of nettle aerial parts were 2-Phenylpropenal (34.1%), the other important compounds were heptanediamide (16.1%), and butanone (12.9%). The ethanolic extract of nettle root powder contained Isothiocyanato-acetaldehyde dimethyl acetal (44.6%)

Table 2. Gas chromatography–mass spectrometry analysis of different parts of nettle powder.

Solvent	Part	Rt	%	Chemical composition	Medicinal effects (Fatthi et al. 2016)
Aqueous	Root	5.8	6.2	2,4-Heptadien-6-ynal	Prostate cancer Anti-inflammatory Hypotensive
		7.5	8.1	2H-1,4-Benzodiazepin-2-one	
		8.4	1.5	2,5-Furandicarboxaldehyde	
		9.8	25.6	3,4,5-Trihydroxybenzoic acid ethyl ester	
		11.8	14.5	Silane	
Aqueous	Aerial	13.8	4.5	Benzeneacetic acid	Anti-diabetic Thrombosis Atherosclerosis Breast cancer Antimicrobial Hypotensive Cardiovascular disease Antiulcer Analgesic T lymphocyte proliferation Antioxidant
		15.7	12.1	Benzaldehyde	
		16.9	13.5	4-[Benzylamino]benzo-1,2,3-triazine	
		17.6	13.7	Benzene	
		3.6	0.2	Butanedioic acid	
		3.8	0.2	2-Methyl-2H-pyrazole-3-carboxylic acid	
		5.7	1.2	2,3-O-Benzal-d-mannosan	
		5.8	1.9	Benzaldehyde	
		6.2	12.9	Butanone	
		7.1	0.6	E-2-Hexenyl benzoate	
		7.6	2.8	Furandicarboxaldehyde	
		8.1	0.2	Monosodium rifamycin SV	
		8.5	34.1	2-Phenylpropanal	
		8.9	4.1	Cyclopropanecarboxamide,	
		9.6	16.1	Heptanediamide	
Ethanollic	Root	9.9	4.1	Benzeneethanol	Anti-prostate cancer Aromatase inhibitor
		10.3	3.1	Benzoic acid	
		10.9	3.4	Cinnamaldehyde	
		12.1	0.8	Phthalic acid	
		15.3	1.3	Phenanthrene	
		15.8	1.3	Benzofuran-2-one	
		16.4	1.5	Benzene	
		17.1	3.9	Naphthalene	
		19.1	2.1	Ethanone	
		22.9	3.3	beta.-Phenylpropiophenone	
		3.2	14.4	Manganese, acetylpentacarbonyl	
		3.5	44.6	Isothiocyanatoacetaldehyde dimethyl acetal	
		8.9	5.7	Imidazole	
Ethanollic	Aerial	10.9	18.9	Acetic acid	Rheumatoid arthritis Allergic rhinitis Antimicrobial Antifungal Antioxidant Anti-helminthic Anti-diabetic Cardiovascular disease
		12.2	5.7	Phthalan	
		14.3	4.5	Tetrazole	
		15.1	6.2	Benzeneethanamine	
		3.4	3.1	Pentenal	
		13.4	5.7	Naphthalene	
		14.3	5.3	2-Propenal	
		15.3	5.1	4-Mercaptophenol	
		15.4	6.2	2-Furancarboxaldehyde	
		16.1	74.4	Compounds contain a simple bond	

Rt: retention time.

and acetic acid (18.9%), Manganese acetylpentacarbonyl (14.4%), while the oil of aerial parts NP contained Naphthalene (5.7%), 2-Propenal (5.3%), 4-Mercaptophenol (5.1%) but the main contents were the compounds does not contain multiple bonds, double or triple bonds (74.4%).

Supplementation of quail diets with 0.5, 1, or 1.5% of NP increased egg production and daily feed intake in comparison to control. As the percent of NP

increased from 0.5 to 1.5% of diet, egg production increased ($p < .0001$) by 67.57, 71.31, and 70.52%, respectively compared to the control (Table 3). The hen day egg production (HDEP, calculated from the 54 weeks of age) slightly increased until the 56 weeks in the experimental groups (67.85, 68.88, and 68.8% in groups NP0.5, NP1, and NP1.5, respectively) compared to the control group (65.3%), but the difference was not statistically significant (Figure 1). However, HDEP

Table 3. Effect of nettle powder on production and reproductive performance in old quails.

Parameter	NP0	NP0.5	NP1	NP1.5	SEM	p-Value
Egg weight (g)	12.30	12.10	12.20	12.20	0.41	.237
Egg production (%)	62.80 ^c	67.60 ^b	71.30 ^a	70.50 ^a	0.40	<.0001
Daily feed intake (g)	24.90 ^b	25.50 ^{ab}	25.70 ^{ab}	26.10 ^a	0.12	.0171
FCR	4.070 ^a	3.91 ^{ab}	3.710 ^c	3.82 ^{bc}	0.03	.0004
Yolk (%)	33.20	33.10	32.80	32.80	0.25	.9
Albumen (%)	56.63	55.78	53.99	55.20	0.58	.43
Egg shell thickness (mm)	0.16 ^b	0.16 ^b	0.17 ^a	0.16 ^a	0.0004	<.0001
Yolk colour	4.82	5.00	5.36	5.29	0.12	.35
Hugh unit	81.80 ^b	82.80 ^{ab}	83.50 ^a	83.60 ^a	0.16	<.0002
Fertility rate	56.40 ^b	60.20 ^{ab}	57.10 ^b	61.70 ^a	0.62	.005
Hatchability of set eggs (%)	46.90 ^b	52.50 ^a	50.00 ^{ab}	53.80 ^a	0.67	.001
Hatchability of fertile eggs (%)	83.20 ^b	87.10 ^a	87.40 ^a	87.00 ^a	0.42	.0006
Embryonic mortality (%)						
Early dead	3.88	2.59	2.77	3.35	0.51	.807
Middle dead	1.35	2.06	1.34	1.60	0.38	.90
Late dead	7.85	5.63	4.97	4.62	0.59	.217
Total mortality	13.10 ^a	10.30 ^b	9.08 ^b	9.57 ^b	0.43	.0003
Ovary (%)	2.19 ^b	2.45 ^{ab}	2.48 ^{ab}	2.80 ^a	0.08	.049
Oviduct (%)	2.34	2.31	2.30	2.36	0.09	.994
Number of HF	2.43	2.86	2.71	3.00	0.16	.685
Number of large YF	2.14 ^b	2.57 ^{ab}	3.43 ^a	3.00 ^{ab}	0.17	.043

^{a-c}Means within rows with different lower case letters are significantly different ($p < .05$).

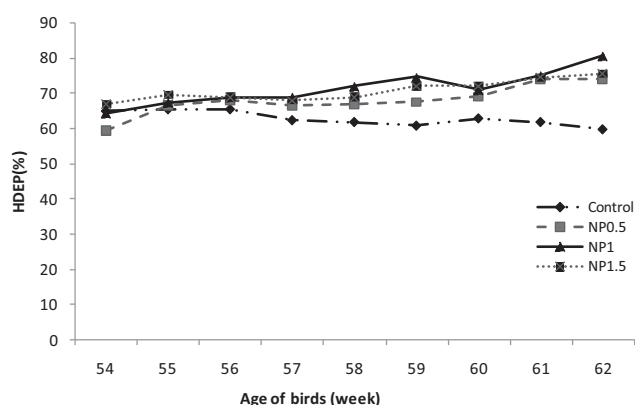


Figure 1. The effect of nettle powder (NP) on hen day egg production (HDEP). Egg production was recorded daily from week 53 to 62 week of age and HDEP was calculated on weekly basis. Values are means, $n = 7$ replicate cages of 4 hens for each replicate.

significantly increased more quickly in dietary nettle powder supplemented groups, when quails were between 57 and 62 weeks of age than in the control group (Figure 1, $p < .05$ and $p < .0001$ at the 57th–58th and at the 59th–62th weeks, respectively). In the same way, at the end of the experiment (when quails were between 59th and 62th week), this parameter remained elevated in the supplemented groups and significantly decreased sharply in the control birds ($p < .0001$). The hen day egg production was the highest in the experimental group receiving 1% nettle powder supplementation (Table 3 and Figure 1), although no dose-effect relationship was evidenced. A similar trend was observed in daily feed intake

Table 4. Effect of nettle powder on some biochemical, hormonal, and oxidative stress parameters of in old quails (62 week of age).

Parameter	NP0	NP0.5	NP1	NP1.5	SEM	p-Value
Oestrogen (pg/mL)	0.65 ^a	0.62 ^{ab}	0.57 ^{ab}	0.49 ^b	0.020	.022
Progesterone (ng/mL)	0.07	0.06	0.11	0.10	0.021	.794
Testosterone (ng/mL)	0.50	0.56	0.64	0.63	0.029	.343
LH (mIU/mL)	0.63 ^b	1.54 ^{ab}	1.49 ^{ab}	1.89 ^a	0.167	.04
FSH (mIU/mL)	2.88	3.54	4.05	3.78	0.363	.724
SOD (unit/mL)	133.70	141.60	149.70	147.00	2.697	.157
CAT (unit/mL)	3.97	4.68	5.18	5.22	0.226	.171
GPX (unit/mL)	161.20	165.20	158.20	163.90	2.414	.769
MDA (nmol/m)	4.88 ^a	4.10 ^b	3.83 ^b	3.86 ^b	0.096	<.0001
TAC (mmol/L)	5.11 ^c	6.20 ^{bc}	7.24 ^{ab}	8.02 ^a	0.270	<.0001
HDL-cholesterol (mmol/L)	2.89	3.29	3.44	3.48	0.240	.275
LDL-cholesterol (mmol/L)	3.41	2.80	2.81	2.61	0.160	.380
Total cholesterol (mmol/L)	5.55 ^a	4.46 ^{ab}	4.25 ^b	3.49 ^b	0.230	.014
Triglycerides (mmol/L)	3.46 ^a	2.89 ^{ab}	2.14 ^b	2.47 ^{ab}	0.150	.008

SOD: superoxide dismutase; GPX: glutathione peroxidase; CAT: catalase; MDA: malondialdehyde.

^{a-c}Means within rows with different lower case letters are significantly different ($p < .05$).

($p < .05$) by increasing NP levels. Feed conversion ratio (FCR) decreased linearly ($p < .05$) by increasing NP levels compared to the control group. There was no significant difference in egg weight between the experimental groups (Table 3). Dietary nettle powder did not cause any significant differences in yolk and albumen percentage and yolk colour. Eggshell thickness improved significantly ($p < .05$) by 1 and 1.5% NP in a dose-dependent manner. Compared with the control, HU was found to increase ($p < .0001$) by 1 and 1.5% NP (Table 3). In relation to reproductive

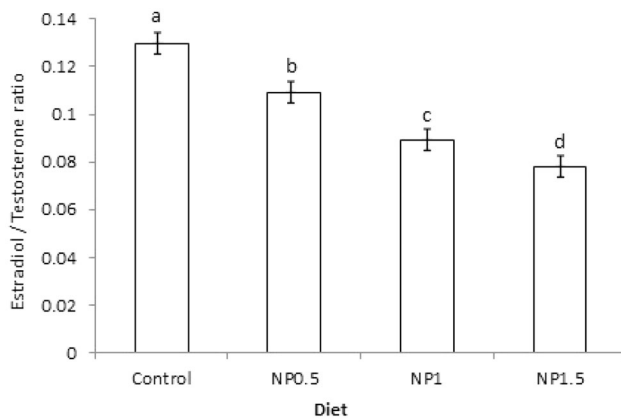


Figure 2. Effect of nettle powder (NP) on Oestradiol to Testosterone ratio in plasma levels of hens at 62 week of age. Control: basic diet without NP; NP0.5: control with 0.5% NP; NP1: control with 1% NP; NP1.5: control with 1.5% NP. ^{a,b,c,d}Mean values with different letters were significantly different ($p < .05$).

performance, fertility rate, hatchability of set eggs and hatchability of fertile eggs increased ($p < .005$) in quails that received nettle powder than the control. The result of this experiment showed that total mortality (%) in fertile eggs decreased ($p < .001$) by 10.28, 9.08, and 9.58 for NP0.5, NP1, and NP1.5 groups, respectively in comparison to the control; 13.07% (Table 3). Data in Table 3 shows that a higher weight of ovary ($p < .05$) at the end of the experiment in layers that received 1.5% NP while the poorest ovary weight belonged to the control group. The numbers of large yellow follicles were increased ($p < .05$) significantly by supplementing NP in the diet.

The concentration of reproductive hormones at the late laying period of experimental quails is presented in Table 4. Dietary supplemented with nettle powder (0.5, 1, and 1.5%) for 10 weeks decreased ($p < .05$) linearly oestrogen level from 0.65 pg/mL in control to 0.62, 0.57, and 0.49 pg/mL, respectively. As well as, the results demonstrate that NP significantly enhanced the levels of LH hormone in the highest level (1.89 mIU/mL) in comparison to control (0.63 mIU/mL). Progesterone, testosterone, and FSH concentrations just differed numerically.

As shown in Table 4, the total antioxidant capacity in plasma increased ($p < .0001$) in a dose-dependent manner of NP compare to control. MDA concentration in all three levels of NP was significantly decreased ($p < .0001$).

Plasma lipid parameters in quails fed at the late laying period were influenced by NP (Table 4). Results indicated that 1 and 1.5% NP in the diet reduced

($p < .05$) significantly plasma levels of triglycerides and total cholesterol compared with the quails fed the basal diet. HDL-cholesterol and LDL-cholesterol didn't affect significantly by NP (Table 4).

Discussion

Herbal medicine, a well-known group of feed additives, is commonly used in the livestock sector specially in poultry production due to beneficial effects such as easy access, low cost, no residual effects, and no antibiotic resistance problems (Attia et al. 2011; Attia et al. 2017; Alagawany et al. 2020). To our knowledge, most of the previous studies on herbal medicine investigated in early stages of the laying period but there is a scarcity of reports on the late laying period for aged animals specially on reproductive performance.

Following the use of different levels of nettle powder (0.5, 1, and 1.5%) for nine weeks, egg production increased 4.76, 8.5, and 7.71%, respectively more than the control group. FCR improved by nettle that could be related to increased efficiency of feed utilisation. It has been proven that phenolic compounds of nettle have considerable antimicrobial and antifungal activity (Bedford 2000; Testai et al. 2002; Gülcin et al. 2004; Loshali et al. 2019). It was reported earlier that using 2% of NP increased feed intake and egg production in hen layers (Mansoub 2011). Alike, Ghasemi and Taherpour. Ghasemi et al. (2014) reported a significant effect in the FCR by using 2% of NP in broiler chick's diet.

Previous studies showed that there is a linear correlation between egg quality and the age of hens (Attia et al. 1994; Burke and Attia 1994). As bird's age increases, larger eggs, and lower shell quality result in easily broken eggs (Roland 1979). Improved eggshell thickness and HU in our study are coinciding with the other investigations on the late laying period (Sahin et al. 2007; Abdel-Wareth and Lohakare 2014). Mansoub (2011) reported that 1.5 and 2% NP improved egg thickness of laying hens but did not have a significant effect on yolk and albumen weight. Abdel-Wareth and Lohakare (2014) demonstrated that dietary supplementation of peppermint at the late laying period of layer hens significantly improved egg thickness and HU but there were no effects on yolk and albumen percentage. Adequate calcium content in nettle could provide a better dietary source of calcium for the eggshell formation and shell quality and possibly can provide safety and stability of eggs (Roland and Bryant 1994). Further, it is reported that

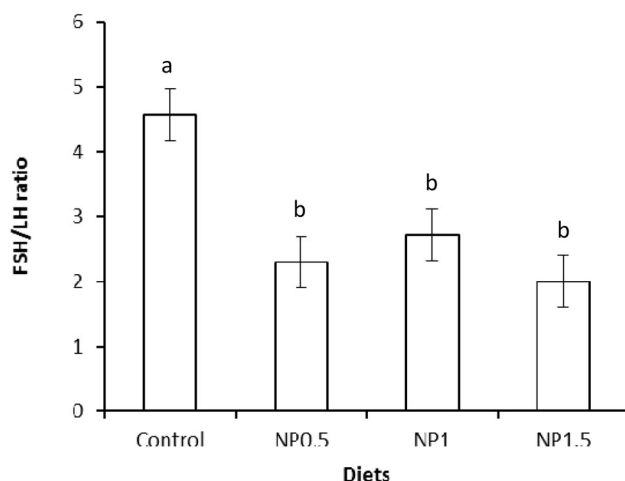


Figure 3. Effect of nettle powder (NP) on FSH/LH ratio in quails at 62 week of age, $p < .001$, $n = 7$ in each group. Control: basic diet without NP; NP0.5: control with 0.5% NP; NP1: control with 1% NP; NP1.5: control with 1.5% NP.

the active component of plants by antimicrobial traits possibly could increase shell weight and shell thickness by providing a healthier environment in the uterus. In contrary to our results, Moula et al. (2019) reported eggshell thickness and HU had no influence by nettle supplementation in the diet at an early stage of laying in quails, which could be due to the influence by different ages, different areas, and source of diet.

The functions of herbal medicine on reproductive parameters have been widely studied in mammals and the researches on poultry especially in females are limited. Both male and female are responsible for infertility (Ottinger 2001; Ottinger et al. 2004). Our results are agreeing with Zhao et al. (2011) who reported hatchability and fertility in laying Japanese quails were improved significantly by a diet supplemented with ginger root.

Ovaries and especially follicles are the most important organs that degenerate by age (Lebedeva 2010). Egg production decreases, coinciding with a decrease in follicular development in aged laying hens (Williams and Sharp 1978a, 1978b). Our data suggested a sharp increase in the number of large yellow follicles and ovarian weight with an increasing percent of NP in the diet of old hens. As hypothesised, we observed a correlation between the serum oestrogens levels and nettle powder percent in bird diets. Since the nettle plant has the potential to inhibit the aromatase and interfere with the conversion of testosterone into oestrogens, therefore the ones receiving 1–1.5% of NP had the serum oestradiol level 0.57 and 0.49 pg/ml, which were 8 and 14% lower than the control hens. Although, there is no difference among the mean

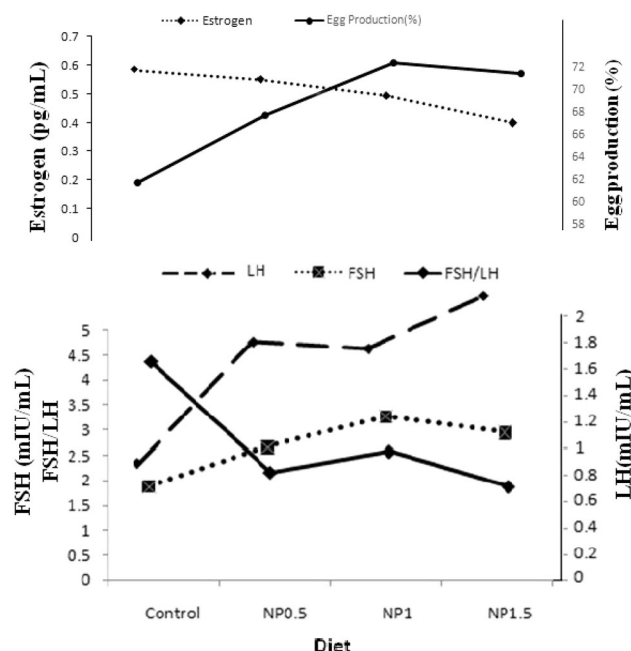


Figure 4. Interplay and comparison of hormones and egg production between experimental groups of quails at 62 week of age. Control: basic diet without NP; NP0.5: control with 0.5% NP; NP1: control with 1% NP; NP1.5: control with 1.5% NP.

values of the serum testosterone level between the different treatment groups. The results of the statistical analyses show significant differences in oestradiol to testosterone ratio (E/T) between experimental hens that received the nettle powder as compared with the control group (Figure 2). Oestradiol/testosterone ratio can be useful in the diagnosis of sexual dysfunction and disorders in males. Some reports have shown that the oestradiol/testosterone ratio has more influence over fertility than does oestradiol alone (Castelló-Porcar and Martínez-Jabaloyas 2016). Even though, a correlation between the E/T ratio and sexuality has been described for a male before, but in this study, we have provided for the first-time data on oestradiol/testosterone ratio that influences reproductive performance in female birds. As the nettle plant dry powder was increased in diet (0.5, 1, and 1.5%) as a source of aromatase inhibitor that can reduce the oestradiol level and increase the level of testosterone, then the E/T ratio reduced 15.8, 31.2, and 39.9% as compared to this ratio in the control group. As a new finding in this study, the egg production increases coincide with the reduction of the E/T ratio in hens (Figure 4). Several investigators reported the correlation of FSH/LH Ratio and LH concentration in predicting pregnancy outcome in humans (Barroso et al. 2001; Ho et al. 2005; Shrim et al. 2006; Liu and Greenblatt 2008; Kofinas and Elias 2014). According to these reports,

we decided to measure the FSH/LH ratio as an independent predictor of reproduction performance for the first time in birds. The analysis of FSH/LH ratio measurements shows a significant difference between the control group and treatments ($p < .01$). The FSH//LH ratio was 4.57 for the control group and $\text{FSH//LH} \leq 2.7$ in NP groups (Figure 3). The $\text{FSH/LH} = 2$ was the lowest ratio in NP1.5 group. In the present experiment, the FSH/LH ratio was strongly correlated with the serum LH concentration and had a lower correlation with the FSH concentration (Figure 4). Our results suggest that the FSH to LH ratio as a valuable index is associated with egg production. Also, FSH/LH ratio showed a stronger correlation with the serum oestrogen concentration (Figure 4). This may then be identified as an interesting conclusion from the work that the Follicle-stimulating hormone/luteinizing hormone ratio = 2 is associated with higher rates of egg production in hens. Also, the serum oestrogen levels and the FSH-to-LH ratio were inversely correlated to the fertility rate of eggs and the number of large yellow follicles in aged birds.

It was concluded that hormonal imbalance for testosterone, oestrogen, follicle-stimulating hormone, and luteinizing hormone is just a major suspected etiologic factor in causing low production in aged birds. It is recommended to achieve a comprehensive case-control study for evaluating hormonal imbalance of (FSH, LH, prolactin, oestrogens, progesterone, and testosterone) hormones in both aged male and female birds. Two previous studies have shown an association between elevated FSH/LH levels and reproductive disorders in females but these reports were limited in human. Liu and Greenblatt (2008) found poorer IVF cycle results even with follicle-stimulating hormone to luteinizing hormone ratio >2 and FSH/LH ratio >3.5 had no positive pregnancy tests. Ho et al. (2005) confirmed that the women with FSH//LH ratio ≥ 3 had a lower mature oocyte number. Several studies have confirmed the suppressor effects of oestrogen on FSH and LH levels during the follicular phase in females. It has been suggested that the FSH and LH are equally sensitive to the suppressing effect of oestradiol (Messinis and Templeton 1990; Messinis 2006). It is well-known that low-level oestrogen imbalance defines the gonadotropin secretions in the female by reducing hypothalamic releasing of GnRH (Shaw et al. 2010). There are now significant evidence to emphasis that the reproductive hormones undergo significant changes with ageing animals (Wise et al. 1997; Yin and Gore 2006; Shaw et al. 2010). It has been shown that the LH and FSH responses to GnRH were

attenuated in older compared with younger females. The oestrogen has an inhibitory effect on FSH releasing was significantly attenuated with ageing. Unlike FSH, the effect of oestrogen on LH amplitude was not significantly changed by ageing (Yin and Gore 2006; Shaw et al. 2010).

It has been proven omega-3 unsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the most abundant fatty acids in nettle leaves, are FSH stimulates which is leading to an increase in blood flow to the ovary to elevate follicles growth and consequently enhance ovarian weight (Bender et al. 2010; Đurović et al. 2017). An alteration of the ovarian activity has a close connection with endocrine system changes, primarily with changes in circulating levels of gonadotropins and sex steroids (Burger et al. 2002). Decreased age-related progesterone, testosterone, LH, and increased oestrogen causes hormonal imbalance status which primarily influences reproductive organ's function (Weil et al. 1999; Vermeulen et al. 2002; Lebedeva et al. 2010; Araujo and Wittert 2011; Walker et al. 2013). Our results showed because of aromatase inhibitor property, NP has the potential to decrease oestrogen levels and consequently increase LH and FSH levels that resulting in an improvement in fertility and egg production in birds. The plasma concentration of progesterone, testosterone, and FSH just increased numerically. During ageing, increased oestrogen with negative feedback on gonadotropins and decrease in preovulatory LH surge results in a reduction in hypothalamic response to gonadal steroids and consequently irregular egg-laying (Ottinger et al. 2004). Wang reported that flavonoids could increase the level of LH by inhibiting aromatase enzymes in the anterior pituitary (Wang et al. 1994). The inhibition of aromatase enzyme prevents oestrogen biosynthesis and thereby releases the hypothalamus-pituitary axis from the negative feedback effect of oestrogen on LH and FSH. Our result is agreeing with Leder et al. (2004) that reported anastrozole (anti-aromatase) even at low doses can increase serum LH levels strongly while decreasing serum oestrogen levels in elderly men. Due to aromatase inhibitors compound [(E10, Z12)-9-hydroxy-10,12-octadecadinoic acid] in nettle extract, LH increasing and oestrogen reduction could be expected (Ganßer and Spittler 1995). One of the most important reasons for ageing is the accumulation of free radicals due to oxidative modification of biomolecules (Fusco et al. 2007). Endogenous antioxidant enzymes like SOD, CAT, and GPX, and MDA are the main antioxidant parameters and act as an indicator for free

radical levels (Premkumar and Agarwal 2012). NP levels could increase total antioxidant capacity at the late laying period of quails. Sharma et al. (2018) found that water extract of nettle, as a source of natural antioxidants in pharmacology and medicine had powerful antioxidant activity against various oxidative systems. Phenolic compounds, melatonin, β -carotene, and vitamins like vitamin C and vitamin E appear to be responsible for the antioxidant activity of nettle (Kratz et al. 2016; Fattah et al. 2017). The concentrations of total cholesterol and triglyceride in the plasma were affected significantly by 1 and 1.5% NP in comparison to the control group. Moula et al. (2019) reported that NP supplementation to the quail diet reduced significantly serum total cholesterol and serum triglyceride levels in Japanese quails. Phytosterol and phenolic compounds such as stigmasterol and campesterol have cholesterol lowering effects (Mavi et al. 2004; Fattah et al. 2017). In the end, although there were two reports that evaluated the effects of dietary supplementation of stinging nettle powder (SNP) on laying performance in quail and Hy-line hens, they demonstrated that the supplementation of SNP to the diet did not influence bird's performance (Mansoub 2011; Moula et al. 2019). One of the reasons of these different results can be because of age of birds. They use the young birds or in peak of production that may have no hormonal imbalances yet but we used the aged birds that confirmed the high imbalances in hormones.

Conclusion

This study demonstrates that the nettle (*Urtica dioica*) had positive impacts on aged quails during the late laying period. Best results were obtained by the addition of 1 or 1.5% NP as evidenced by improved egg production, FCR, egg-shell thickness, fertility, hatchability, weight of ovary, number of follicles, hormones, and antioxidant situation. Our results suggest that the *Urtica dioica* has a regulative effect on sex hormones imbalance in old birds.

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Disclosure statement

The authors declare no conflicts of interest to report with regard to the compilation and/or publication of this article.

Ethical approval

All applicable institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Kurdistan, Sanandaj, Iran.

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Data availability statement

The datasets generated for this study are available on request to the corresponding author.

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