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IN-OVO INJECTION OF *Citrus aurantifolia* EXTRACT AT DAY 18 OF INCUBATION: EFFECTS ON INCUBATION TRAITS, HATCHABILITY, GROWTH PERFORMANCE, AND HAEMATOLOGICAL INDICES OF ISA BROWN LAYER CHICKS

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ABSTRACT

This study evaluated the effects of in-ovo injection of extract of Citrus aurantifolia administered at day 18 of incubation-on-incubation characteristics, hatchability traits, post-hatch growth performance (to day 28), and haematological indices of ISA Brown layer chicks. A fresh matured Citrus aurantifolia seeds were air-dried at room temperature for 3 weeks and extraction was done using water and ethanol. A total of 360 fertile eggs (120 eggs per treatment; 3 replicates of 40 eggs each) were assigned to three treatments: uninjected control (0 µg/ml; T1), 0.4 µg/ml extract (T2), and 0.6 µg/ml of Citrus aurantiifolia seed extract (T3). Percentage egg weight loss (PEWL) was determined between 0–12, 12–18, and 0–18 d of incubation. Hatchability, chick yield, hatch residue analysis, growth performance, and day-old haematology were evaluated. Data were subjected to analysis of variance. There was significantly ($P \leq 0.05$) reduction of PEWL across incubation periods. Whereas hatchability and chick yield were not significant ($P > 0.05$). Chicks from T3 showed improved feed conversion ratio (FCR) and higher packed cell volume (PCV), haemoglobin (Hb), and red blood cell (RBC) counts. However, dead chick percentage increased at the higher dose. These findings indicate that low-volume in-ovo administration of C. aurantifolia extract (CASE) can enhance physiological status and feed efficiency of ISA Brown chicks, although proper dose adjustment is needed.

Keywords: In-Ovo Injection, *Citrus Aurantifolia*, ISA Brown, Hatchability, Haematology

INTRODUCTION

Modern commercial layer and broiler chicks experience delays in accessing feed and water until they reach the shelter, especially during their critical first period of life. This means that they spend 4.5–10% of their lives without feed and water (Kanagaraju, 2014) until after 24–36 h after hatching, which causes the mobilization of body reserves to support metabolism, resulting in decreased body weight, impairing overall growth, immune sufficiency and health performance (Kanagaraju and Rathnapraba, 2019).

Hatchery objectives are to obtain high hatchability with day-old chickens of optimal quality to maximize profitability while commercial farms prefer high-quality chicks as key determinants of a successful and profitable production cycle (Narin¸c, 2022). Improving hatchability, chick quality, and performance will mean additional profit for hatchery and farm managers (Kuka *et al.*, 2023).

In ovo injection is an emerging technique that provides the opportunity to deliver nutrients, probiotics, or medicinal plant extracts directly into the egg during incubation. In ovo feeding of substances such as antioxidants during incubation may improve the antioxidant status of the chicken embryo and post hatch growth phases (Yigit *et al.*, 2014; Elsaadany, 2019). Moreover, in-ovo feeding of chickens with extracts from numerous plant products has enhanced their defenses against the contagious bursal virus, avian influenza virus, and fowl pox virus (Sood *et al.*, 2013; Nyandoro *et al.*, 2014) The antioxidant level of the chicken embryo may be enhanced by in ovo injection of antioxidants because they have an effective defense against free radicals (Salary et al., 2014). Recently, attention has been shifted to the use of herbal additives as growth promoters and antioxidant components from herbs, spices, and their products (Tokofai *et al.*, 2020; Kpomasse *et al*., 2021; Adjei-Mensah *et al.*, 2022; Kpomasse *et al*., 2023). Though the results are promising, there exist some contradictions mainly due to the varying nutrient compositions of the substances, extraction techniques employed, the dose and the moment of injection (N'nanle *et al.*, 2017; Oke *et al*., 2021; Sogunle *et al*., 2022; Adjei-Mensah *et al*., 2022), which necessitate the exploration of more materials, especially plant materials to validate the in ovo injection approach. Plants are a rich source of essential nutrients (Samtiya *et al*., 2021), chemicals and other active components (Radha *et al*., 2021).

The seeds in citrus fruits are classified as part of the citrus waste. According to Nobakht (2013), the seeds of citrus fruits contain active antioxidants, including a mixture of flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins and epicatechins. Lime (*Citrus aurantifolia*) seed contains approximately 8.78% crude protein, 37.95% carbohydrate and 5.01% fiber (Boubekri, 2014). Hence, it is hypothesized that injecting lime seed extracts into broiler eggs during prehatch will enhance the hatching profile and growth performance as well as improve the health and ameliorates heat stress of birds. Therefore, the current study evaluated the effects of in ovo feeding of *Citrus aurantifolia* seeds extract during incubation period.

MATERIALS AND METHODS

Experimental Design

A total of 360 Isa brown breeder eggs with an average weight of 56.4±1.2g from a 48-wk-old flock from the National Veterinary Research Institute (NVRI) were used for this study. The eggs were incubated at standard incubation conditions including a temperature of 37.5°C, relative humidity of 50 to 60% and turning once every 90 min until d 18 of incubation in a locally made solar/gas incubator. At d 18 of incubation, the incubated eggs were candled and the eggs with evidence of living embryos were divided into 3 groups of 120 eggs each. The groups were divided into 3 replicates of 40 eggs and each replicate was in a hatch basket. The baskets were randomly put in the hatcher. These groups were (1) Control: Eggs without injection, (2) Ext 0.4mg: eggs injected with 0.4mg/ml of CASE and (3) Ext 0.6mg: eggs

injected with 0.6mg/ml of CASE. The injection volume for all groups was 0.1ml/egg.

***Citrus aurantiifolia* Seeds Extraction**

Citrus aurantifolia seeds were collected from fresh matured and unaffected fruits and air-dried at 30˚C for 3 wk. The dried seeds were pulverized into powder using a laboratory crusher equipped with a 10 mm mesh sieve and weighed. The extraction was done with water and ethanol. A mass of 400 g of crushed *Citrus aurantifolia* seeds was dissolved in 4 l of an ethanol-water mixture (70/30, v/v). The mixture was brought to room temperature and macerated with stirring for 72 h then the recovered extract was filtered. The filtrate obtained was evaporated in a rotary evaporator at 45˚C. The extract was then placed in a Petri dish in an oven at 40˚C until drying (Boubekri, 2014) to obtain 112 g of hydro-ethanolic extract.

***Citrus aurantiifolia* Seeds Extract Injection**

Approximately, 20 ml of saline solution was added to 10 mg of CASE and homogenized with a vortex to obtain a 500 ug/ml solution. After successive dilutions, a series of injectable solutions containing 0.4 µg/ml, and 0.6 µg/ml were obtained. Injection of CASE was made in the air chamber of the living embryo grouped in treatment during d 18 of incubation. A needle was utilized to create 2 holes in the shell, specifically above the air chamber. This was done to reduce the pressure inside the chamber and aid in the retention of the injected solution. The injection volume for all groups was 0.1 ml per egg and was administered within the air cell using insulin syringe equipped with a very thin needle. After injection into 1 of the holes, both holes were sealed with wax and the egg was placed in the hatching baskets.

Hatching Event

Beginning from 18 until 22 days of incubation, eggs were observed every 3 h individually for hatching events such as internal piping, external piping, and chick emergence. The following parameters were evaluated for each treatment according to Zhong et al. (2018).

Embryonic mortality (%)

$$= \frac{\text{number of dead embryos}}{number\ of\ fertile\ eggs}\ x\ 100$$

Pipped but unhatched (%)

$$\frac{\text{number of pipped but unhatched}}{number\ of\ fertile\ eggs}\ x\ 100$$

Chick yield (%)

$$= \frac{\text{chick weight at hatch}}{initial\ egg\ weight}\ x\ 100$$

Egg weight loss (%)

$$= \frac{\text{initial egg weight} - \text{egg weight at transfer to hatcher}}{initial\ egg\ weight}\ x\ 100$$

Pipped live (%)

$$= \frac{\text{pipped live}}{number\ of\ fertile\ eggs}\ x\ 100$$

Dead chicks (%)

$$= \frac{\text{dead chicks}}{hatched\ chicks}\ x\ 100$$

Hatchability (%)

$$= \frac{\text{number of hatched chicks}}{\text{number of fertile eggs}}\ x\ 100$$

The incubator was stopped at 22 days and unhatched eggs were cracked, classified as “infertile eggs” (candling error) and eggs with dead embryos and hatchability were expressed as the percentage of fertile eggs.

Post Hatch Performance

According to each treatment, the hatched chicks were divided into 3 replicates of 15 birds and were assigned to a deep litter system. Chickens were reared for 3 wk. In this period, breeding conditions were the same for all chicks in terms of temperature changes and vaccination time and free access to feed and water were provided. Commercial feeds were used in this experiment for all experimental groups. Weekly, body weight and feed intake of each replication were recorded to determine the feed conversion ratio and weight gain of each treatment correct for mortality.

Serum Haematological Analysis

The blood samples were collected, placed in EDTA tube and analyzed for the following parameters: Hemoglobin, white blood cell (WBC), red blood cell (RBC), Neutrophil, and lymphocyte using an automatic analyzer.

Growth Performance

Growth performance such as body weight gain and feed intake parameters was recorded. This was measured weekly from 1-21 days (3 weeks). The feed intake was measured based on the quantity of feed that had disappeared over 24 hrs in the feeder.

RESULTS AND DISCUSSION

The hatch variables, including 0 to 12, 12 to 18, and 0 to 18 doi PEWL; hatchability percentage; and chick yield, are presented in Table 1. No significant ($p > 0.05$) treatment difference were observed in percentage hatchability and chick yield whereas considerable ($p > 0.05$) differences were noticed in PEWL 0 to 12, 12 to 18, and 0 to 18 doi. The injection of 0.4 µg/ml and 0.6 µg/ml CASE did not exhibit negative effects on the hatchability and chick yield of the layer chicks. Similar to the current study, the in ovo injection of saline containing 0.5, 1.5, 4.5, or 13.5 mg of L-AA has been previously observed to not affect the hatchability or hatchling BW of broilers Mousstaaid *et al.,* (2022). This indicates that a higher in ovo injection dosage of 0.6 µg/ml CASE of the current study is safe. Dissimilar to the current findings, it has been reported that the in ovo injection of 3 mg of L- Ascorbic Acid (L-AA) at 11 and 15 doi increased hatchability Zhu *et al.,* (2021). Inconsistencies between the results of the current and previous research may be linked to the time and site of injection, the mode of injection, or the level of injected phytogenic extracts. The significant ($p< 0.05$) difference observed in PWEL in the present studies is in contrary with the studies conducted by Mousstaaid *et al.,* (2022) who observed similar values in eggs injected with saline and L-AA solutions. The reduction in egg weight loss suggests improved embryonic metabolic regulation.

Table 2 showed the effect of In-ovo injection of *Citrus aurantiifolia* extract on hatch residue analysis variables. Late, pip, post-pip, and hatchling mortalities were defined, respectively, as those mortalities that occurred between 18 and 21 doi prior to pip, during the pipping process, after the pipping process, and immediately after complete emergence from the shell. Hatch residue analysis revealed that late, pip, and post-pip mortalities did not differ significantly ($p > 0.05$) among treatments. It could be noticed in this present study that there is no effect of CASE on late dead, pipped dead, pipped live, and dead chicks and this agrees with the study of Mousstaaid *et al.,* (2022) who also reported that there is no influence of L-AA injection of broiler eggs. Dead chick percentage increased at the highest dose.

Effect of In-ovo injection of *Citrus aurantiifolia* extract on the growth performance of chicks is presented in Table 3. There were significant ($p > 0.05$) differences in all the parameters evaluated except for initial body weight where similar values ($p > 0.05$) were recorded. Injection of 0.6 *µg/ml* CASE had the best feed conversion ratio relatively to other treatments.

The results on the effect of in ovo feeding of CASE on the growth performance of layer chicks are presented in Table 3. During the entire rearing period, the increase in the body weight gain and feed conversion ratio of chickens in the Ext 0.6mg group was significantly improved compared to the other treatment groups (P<0.05) and could probably be due to the bioactive components in the extract used which improve digestive enzymes. This result is in line with the finding of N'nanle *et al*. (2017) who stated that Moringa leaves extract injected on d 18 of incubation significantly affected the bodyweight at 6 and 7wk of age of layer chick. This supports the results of Bhanja *et al*. (2012) and Shafey *et al*. (2014) who observed body weight improvement in broiler chickens fed in ovo with vitamins and amino acids. The best values of body weight gain and feed conversion ratio were recorded for birds that received 0.6 mg/ml of CASE.

Table 4 shows the results on the effect of in ovo feeding of CASE on haematological indices. All parameters analyzed showed significant (p< 0.05) differences except red blood cells. The 0.6 *µg/ml* group showed improved PCV, Hb, lymphocytes, and RBC values and this agrees with the findings of Kpossou *et al*., (2024) who reported that the counts of red blood cells and the hemoglobin of the chicks hatched from the eggs that were injected with CASE had a significant increase compared to the control which is also in line with the results of the Panda and Cherian, (2014) who stated in their experimental results that the increase in the hemoglobin concentration of the newly hatched broilers is directly related to the embryo's nutrition during the incubation period. According to a study by Naeem *et al.,* (2022), dehydration and nutrient deficiency can adversely affect the hematopoietic process in broiler embryos. Therefore, the decrease in blood cells in the control group can be attributed to the loss of humidity and insufficiency of nutrients in the stored eggs Campbell *et al.,* (1994).

Table 1: Percentage Egg Weight Loss between 0 and 12, 12 and 18, and 0 And 18 Days of Incubation (Doi) and Hatchability of Injected Live Embryonated Egg

		Treatment		
Parameters	**Control**	**Ext0.4mg**	**Ext0.6mg**	***p*-value**
0-12 PEWL (%)	5.29±0.10[a]	4.50±0.22[b]	3.86±0.07[c]	0.00
12-18 PEWL (%)	4.09±0.03[a]	3.49±0.04[b]	3.20±0.19[c]	0.00
0-18 PEWL (%)	9.16±0.12[a]	7.83±0.23[b]	6.94±1.44[c]	0.00
Hatchability (%)	75.57±3.47	73.17±7.33	71.12±0.67	0.87
Chick yield (%)	71.73±0.37	70.74±0.44	71.23±0.05	0.19

Abbreviations; Control (eggs without injection); Ext0.4mg (eggs injected with 0.4 mg/ml of Citrus aurantiifolia seeds extract, CASE); Ext0.6mg (eggs injected with 0.6 mg/ml of CASE).

[a,b,c] Different letters indicate significant differences between means within rows (P < 0.05).

Table 2: Effect of In-Ovo Injection of *Citrus Aurantifolia* Extract on Hatch Residue Analysis Variables (Late, Pip, Post-Pip, and Hatchling Mortality) At 21 Days of Incubation

		Treatment		
Parameters	**Control**	**Ext0.4mg**	**Ext0.6mg**	***p*-value**
Late dead (%)[1]	24.43±3.47	26.83±7.33	28.87±6.47	0.87
Pipped dead (%)[2]	2.67±0.33	2.33±0.88	3.33±0.67	0.57
Pipped live (%)[3]	0.67±0.33	1.00±0	2.00±0.58	0.11
Dead chick (%)[4]	2.90±1.45	4.32±0.48	8.97±2.47	0.09

Abbreviations; Control (eggs without injection); Ext0.4mg (eggs injected with 0.4 mg/ml of Citrus aurantiifolia seeds extract, CASE); Ext0.6mg (eggs injected with 0.6 mg/ml of CASE).

[a,b,c] Different letters indicate significant differences between means within rows (P < 0.05), [1] Mortality between 18 and 21 doi, prior to pip. [2] Mortality during the pipping process. [3] Mortality after the pipping process. [4] Mortality immediately after complete emergence of hatchlings from the shell. [5] Time of injection = 18 doi.

Table 3: Effect of In-Ovo Injection of *Citrus Aurantifolia* Extract on the Growth Performance of Chicks

		Treatments		
Parameters	**Control**	**Ext0.4mg**	**Ext0.6mg**	***p*-value**
IBW	40.29±0.11	39.92±0.14	40.09±0.07	0.13
FBW	478.86±2.15[a]	468.72±3.17[b]	477.69±1.0[a]	0.04
BWG	438.57±2.20[a]	428.80±3.04[b]	437.60±0.94[a]	0.04
FI	572.00±1.35[a]	580.25±9.28[a]	500.90±4.43[b]	0.00
FCR	1.30±0.01[b]	1.35±0.02[a]	1.15±0.01[c]	0.00

IBW; Initial body weight, FBW: Final body weight, BWG: Body weight gain, FI: Feed intake, FCR: Feed conversion ratio

Abbreviations; Control (eggs without injection); Ext0.4mg (eggs injected with 0.4 mg/ml of Citrus aurantiifolia seeds extract, CASE); Ext0.6mg (eggs injected with 0.6 mg/ml of CASE).

[a,b,c] Different letters indicate significant differences between means within rows (P < 0.05).

Table 4: Effect of In-Ovo Injection of *Citrus Aurantiifolia* Extract on the Haematological Indices of Chicks

	Treatments			
Parameters	**Control**	**Ext0.4mg**	**Ext0.6mg**	***p*-value**
PCV (%)	27.97±0.08^{c}	30.17±0.47^{b}	31.63±0.42^{a}	0.00
Hb (g/dL)	9.23±0.09^{c}	10.09±0.09^{b}	10.50±0.06^{a}	0.00
RBC (x 10^{6}/µL)	2.40±0.02^{c}	2.72±0.03^{b}	2.87±0.02^{a}	0.00
WBC (x 10^{3}/µL)	11.80±0.59	10.70±0.17	11.43±0.47	0.28
N (%)	41.67±0.08^{a}	37.92±0.67^{b}	35.58±0.46^{c}	0.00
L (%)	54.92±0.33^{c}	58.13±0.13^{b}	59.43±0.47^{a}	0.00

Abbreviations; Control (eggs without injection); Ext0.4mg (eggs injected with 0.4 mg/ml of Citrus aurantiifolia seeds extract, CASE); Ext0.6mg (eggs injected with 0.6 mg/ml of CASE).
^{a,b,c} Different letters indicate significant differences between means within rows ($P < 0.05$).

CONCLUSION

In-ovo injection of 0.4–0.6 *µg/ml* extract of *Citrus aurantifolia* at day 18 improves feed efficiency and haematological indices of ISA Brown chicks without adversely affecting hatchability.

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