



Effect of Herbal Extract Mixture Supplementation in Poultry Diets on Growth Performance, Hematological Indices and Fecal Microbial Counts in Ri Crossbred Chicken

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Abstract | The aim of the present study was to evaluate the effect of supplementing the herbal extract mixture (50% of *Tithonia diversifolia*, 20% of *Achyranthes aspera*, 20% of *Pseuderanthemum palatiferum*, and 10% of *Polyscias fruticosa*) on growth performance, hematological indices and fecal *E. coli*, *Salmonella* counts of growing chickens. A total 450 one-day old Ri crossbred chicks were randomly located to 5 dietary treatments with 3 replicates (30 chicks per replicate) namely Negative control (NCTL) in which birds fed a basal diet; Positive control (PCTL): added 1 g colistin per 1 kg of basal diet for first 3 weeks of age; and experimental treatments HE05, HE10 and HE15 in which 0.5 g, 1 g and 1.5 g of herbal extract mixture added in 1 kg of basal diet, respectively. The results showed that, growth performance, hematological indices were not affected by the addition of herbal extract mixtures in feed ($p > 0.05$). Fecal *E. coli* counts changed slightly with the bird age, however addition of herbal extract mixture reduced significantly number of fecal *Salmonella* counts. Further studies are needed to explore the effect of higher herbal extract levels on hematological indices and microbial counts in poultry.

Keywords | Alanine aminotransferase, Herbal extract, *E. coli*, Chicken, *Salmonella*, Hematological indices

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INTRODUCTION

Escherichia coli (*E. coli*) and *Salmonella* are common Gram-negative bacteria and cause gastrointestinal diseases in animals in general, including poultry such as diarrhea and paratyphoid, leading to great losses in the livestock industry; in addition, they are also the cause of food poisoning in humans (Alonso *et al.*, 2011; Newman *et al.*, 2021). Common strategies to prevent these diseases

include the use of vaccines and antibiotics; however, the use of antibiotics has many serious consequences such as antibiotic resistance, reducing the effectiveness of antibiotics and changing the microbiome in intestinal tract and negatively affects health and residues in animal products (Farhat *et al.*, 2023); meanwhile, there is no vaccine that is directly effective in protecting chickens from infection, mainly due to the wide variety of bacterial strains and lack of cross-protection (Mehat *et al.*, 2021). In fact, both *E. coli*

and *Salmonella* have been listed by the WHO as among the pathogens requiring antibiotic susceptibility determination before treatment (WHO, 2024). Consequently, there is a need to develop alternatives to antibiotics, such as probiotics, postbiotics, and herbal medicines, as bacteria are less likely to develop resistance to these natural compounds (Brijesh *et al.*, 2009; Singh, 2013). The utilization of herbal medicines alternatives to antibiotics has become an interest in recent years. Many natural plant products have been supplemented in animal diets to promote growth performance and immunological response (Shim *et al.*, 2012; Liu *et al.*, 2013; Bontempo *et al.*, 2014; Sampath *et al.*, 2020). In addition, the use of herbal extracts not only enhanced disease resistance, reduced the reliance on veterinary drugs, promoted growth but also ensured a stable supply of clean meat for consumers (Camargo *et al.*, 2023; Orimaye *et al.*, 2024).

In Vietnam, many studies on the use of herbal extracts in poultry diets to improve performance, promote intestinal microflora and mitigate fecal *E. coli* and *Salmonella* bacteria have been reported (Nguyen *et al.*, 2022; Nguyen and Nguyen, 2022; Pham and Le, 2023; Le and Nguyen Tuyet, 2023). According to Do Tat Loi (2004), da-quy (*Tithonia diversifolia*), co-xuoc (*Achyranthes aspera*), hoan-ngoc (*Pseuderanthemum palatiferum*), and dinh-lang (*Polyscias fruticosa*) have been used to prevent and treat diseases in humans and animals. Da-quy and Co-xuoc are wild plants with the huge area and free to harvest in the Central Vietnam, meanwhile Hoan ngoc is also a wild plant with limited area. Dinh-lang is cultivated and used as human herbal medicine.

Antioxidant activity is an important feature of herbal plants because antioxidants play a protective role in animal health (Alloui *et al.*, 2014). Antioxidants promote animal health by scavenging reactive oxygen species *in vivo*, thereby preventing cellular damage. Moreover, antioxidant compounds could hold promise for the control of infectious diseases due to the association of infection with lipid peroxidation of the intestinal mucosa (Naidoo *et al.*, 2008). However, the use of medical herbs can also change blood hematological indices (Mwale and Masika, 2009). Because the ingredients of medicinal herbs have effects on some organs in the body such as the liver, hematopoietic organs (Patzer, 2008) and cause effects such as reducing red cell volume, red blood cells, hemoglobin; changing white blood cell indices, increasing total protein, albumin, aspartate transaminase and alanine transaminase (El Hilaly *et al.*, 2004; Oduala *et al.*, 2007; Adedapo *et al.*, 2007).

This study therefore aimed at evaluating the effect of inclusion of the herbal extract combination on growth performance, hematological parameters and fecal *E. coli* and *Salmonella* in Ri crossbred chicken.

HERBAL EXTRACT PREPARATION

Co-xuoc, da-quy, dinh-lang and hoan-ngoc were individually dried at 65°C in a Memmert UN75 oven (Mettler, Germany) for 24 hours after being wilted at room temperature overnight, then ground and stored in vacuum-sealed plastic bags. The individual herbs were extracted using 90% ethanol as described by Wendakoon *et al.* (2012). Briefly, the ratio between the sample and the extraction solvent was 10% and the mixtures were soaked and shaken at 65°C, 30 rpm for 4 hours in a water bath system HD-501 (EMIN, Singapore). Then the extract was filtered to remove the residue and the solvent was removed at 65°C. The solid of the individual herbs was mixed with the substrate at a ratio of 2:1 w/w; then the mixture is dried at 65°C until it becomes a dry powder. Moreover, the extraction temperature (65°C) and solvent concentration (90% ethanol) in the current study was adapted from our preliminary test which show the highest results. The herbal extracts used in the experiment were mixed (DM basic) in powder form between 50% of co-xuoc, 20% of da-quy, 20% of hoan-ngoc, and 10% of dinh-lang by a blender machine. The herbal extracts collected in a powder form from fresh plants of co-xuoc, da-quy, hoan-ngoc and dinh-lang were 21%, 24.7%, 18.7% and 25%, respectively.

FEED INGREDIENT AND BASAL DIET

Chemical composition of feed ingredients presents in Table 1. The basal diet was proportionally formulated using the ingredients to meet the nutrient requirements at different growing stages according to the TCVN2265:2020 and its nutritive value present in Table 2. Briefly, for local chicken, the ME of feed is no less than 2900 Kcal/kg for chicken at 1-4 weeks old and 5-8 weeks old and 3000 Kcal/kg for chicken from 9 weeks olds until finish. Crude protein requirement for chicken at periods of 1-4 weeks old, 5-8 weeks old and 9 weeks old until finish are no less than 20%, 18% and 16%, respectively.

Table 1: Analyzed chemical composition of feed ingredients (% DM).

Ingredient	DM	CP	EE	Ash
Cassava meal	88.3	2.59	3.23	1.60
Corn meal	86.0	8.26	3.64	0.60
Extracted rice bran	88.8	15.9	11.8	15.3
Soybean meal	88.4	50.5	2.29	6.44
Fishmeal	86.8	51.0	4.75	16.6

* All values are expressed as % of dry matter (DM).

EXPERIMENTAL DESIGN

A total of 450 Ri crossbred chickens of 1 day old were randomly assigned to 5 dietary treatments with 3 replicates and in 30 birds (15 male and 15 female) in each replicate.

Five treatments included negative control (NCTL) in which birds fed a basal diet without antibiotic supplementation; Positive control (PCTL): birds fed a basal diet and supplemented with 1 g colistin per 1 kg of feed from 1 to 21 days old, then no further supplementation (following national legislation); and experimental treatments HE05, HE10 and HE15 in which birds fed a basal diet with supplemented 0.5 g, 1 g and 1.5 g of the herbal extracts per 1 kg of basal diet, respectively. The experiment lasted for 100 days.

Table 2: Ingredient proportion and nutritive value of basal diets.

	Growing period (week)		
	1-4	5-8	9- final
Ingredient proportion (g/kg)			
Corn meal	550	440	500
Extracted rice bran	150	200	221
Cassava meal	28	100	70
Soybean meal	220	188	120
Fishmeal	50	71	89.1
Salt	1	1	1
Premix-mineral	2	1	1
Methionine (g/kg)	1.91	0.85	0.2
Lysine (g/kg)	1.05	0.53	-
Calculated nutritive value			
ME (kcal/kg DM)*	3560	3510	3500
DM (%)	89.5	89.1	89.8
CP (% DM)	21.1	19.9	17.8

* Metabolizable Energy (ME): is expressed in kcal/kg DM.

GROWTH MEASUREMENTS

Growth performance data of chickens were collected from 4 weeks old until finish (14 weeks old). In each replicate, six birds (sex equal) in total of 30 birds were randomized selected and marked by number; then these birds were weighted one week-interval to calculate average daily gain (ADG) accordingly. Daily feed offered and refusals were recorded for feed intake (FI) calculation. Then feed conversion ratio (FCR) of each replicate was accordingly calculated by ratio between FI and ADG.

HEMATOLOGICAL INDEX ANALYSIS

Blood samples were collected via wing veins of 4 chickens/replicate (sex equal) in the morning of the day before the end of the experiment. The 2 ml of blood samples from each chicken were taken and deposited in plastic tubes containing anticoagulant. Samples were centrifuged to separate the plasma from the serum for biochemical analysis using the Cobas 6000 Biochemistry – Immunoassay analyzer system (Roche, Switzerland). The parameters measured included creatinine, aspartate aminotransferase;

alanine aminotransferase; bilirubin total; bilirubin-direct, bilirubin-indirect; lactate dehydrogenase; cholesterol; high density lipoprotein cholesterol; low density lipoprotein cholesterol; alkaline phosphatase.

FECAL E. COLI AND SALMONELLA COUNTS

Based on growth phases, fecal samples were collected from 2 birds/replicate at 1st, 14th, 28th, 48th and 99th day of the experiment. The sample was processed by weighing 1 g of sample and mixing with 10 mL of peptone water. This mixture was then diluted 10 times, and each concentration was spread onto 3 plates of Eosin methylene blue agar (EMB; Conda Laboratories, S.A., Spain), with each plate inoculated with 100 µL.

One gram of stool sample in 10 mL of 0.1% w/v peptone water, was stomached for 2 min. A 1 mL portion of each homogenate was used to prepare 10-fold dilutions, to 10⁻⁵, in 0.1% w/v peptone water. Portions of 0.1 mL of the homogenate and each dilution were spread on triplicate plates of Eosin methylene blue (EMB) agar (Conda Laboratories, S.A., Spain). The plates were incubated at 37 °C for 24h, and colony numbers were determined from plates bearing 20 to 200 colonies. The total number of *E. coli* was quantified as colony forming units per gram of sample (CFU/g) by counting all colonies that were dark red to purple with metallic sheen on EMB medium.

The density of *Salmonella* bacteria was performed as described by Yue *et al.* (2014). At each concentration, the diluted sample was spread on 3 plates of xylose-lysine deoxycholate medium (XLD, Merck, Germany), incubated at 37 °C for 24 h. Total *Salmonella* was quantified as colony-forming units per gram of sample (CFU/g) by counting all round, black colonies on XLD medium.

CHEMICAL ANALYSES

The DM contents of feed ingredients and a basal diet were determined by drying at 105°C for 16 to 20 h until constant weight (Association of Official Analytical Chemists 930.15, 1990). Ash content of feed ingredients was determined by drying at 550°C for 3 to 5 h (AOAC 942.05, 1990). Nitrogen concentration in the feed ingredients and a basal diet was determined using the automatic Kjeldahl destruction and distillation machine (Velp Scientifica, Usmate, Italia) following the Kjeldahl method (AOAC 930.15, 1990). Feed ingredients were analysed for ether extract (EE) using the Soxhlet method (Sci Finetech, Seoul, Korea) (AOAC 930.15, 1990).

STATISTICAL ANALYSIS

Data were entered and managed using Microsoft Excel 2016 MSO software (16.0.4266.1001). Descriptive statistics and statistical difference analysis were performed on SPSS 18.0 software (IBM SPSS Statistics version 18.0, IBM,

Table 3: Hematological indices of the blood samples collected at the end of the experiment.

Item [#]	NCTL	PCTL	HE05	HE10	HE15	SEM	p-value
Creatinine (μmol/L)	3.3	5.0	7.0	6.0	3.3	1.76	0.53
AST (SGOT) (U/L)	175.7	215.7	203.0	174.7	188.7	15.35	0.32
ALT (SGPT) (U/L)	4.7	4.3	2.7	1.7	3.3	1.02	0.29
Bilirubin total (μmol/L)	0.9	0.9	0.9	1.7	1.1	0.21	0.11
Bilirubin-Direct (μmol/L)	0.2	0.3	0.3	0.5	0.3	0.12	0.41
Bilirubin-Indirect (μmol/L)	0.7	0.6	0.6	1.2	0.8	0.16	0.14
LDH (U/L)	354.0	362.7	357.3	349.0	316.3	63.67	0.99
Cholesterol (mmol/L)	2.5	2.5	2.4	2.7	2.0	0.21	0.30
HDL-C (mmol/L)	1.6	1.4	1.4	1.4	1.2	0.14	0.43
LDL-C (mmol/L)	0.5	0.8	0.5	0.8	0.8	0.19	0.53
Alkaline phosphatase (U/L)	92.7	63.0	259.3	133.7	187.3	59.38	0.22

[#] **AST:** aspartate aminotransferase; **ALT:** alanine aminotransferase; **LDH:** lactate dehydrogenase; **HDL-C:** high density lipoprotein cholesterol; **LDL-C:** low density lipoprotein cholesterol.

Armonk, NY, USA). Effect of treatments on average daily weight gain, feed intake, feed conversion, hematological indices and microbial counts (log transformation was needed if the data is not normally distributed) were analysed using analysis of variance (ANOVA) and a post-hoc Turkey test to compare the difference between treatments when the p value of ANOVA ≤ 0.05 .

RESULTS AND DISCUSSION

HEMATOLOGICAL INDICES

Hematological indices represent the process of nutrient metabolism in the body and reflect the health status of animals (Hu *et al.*, 2016). There are numeral factors including environmental changes, pathological conditions, feed quality and drug use that affect hematological indices in chickens (Chaturvedani *et al.*, 2017; Basit *et al.*, 2020). Data in Table 3 showed that there was no statistical difference in the blood hematological indices of chickens among treatments ($p > 0.05$). Our findings on some hematological indices were in agreement with previous studies (Rafeeq *et al.*, 2021), who reported that total protein and alanin aminotransferase (ALT) were not affected by adding 200 mg/kg feed of cumin (*Cuminum cyminum*) or jir (*Artemisia scoparia*) on diets of broiler chickens. Rafeeq *et al.* (2021) found the positive effect of adding cumin and jir in diets on Red Blood Cell and White Blood Cell. In our study, due to technical limitation, some serum biochemical compositions such as red cell, white cell, hemoglobin, albumin, globulin, etc. could not be analyzed. In addition, the hematological indices of Ri crossbred chickens in this study were lower than those as compared to other chicken breeds (Mroczek *et al.*, 2013). In general, the dosage of herbal extracts use in the current experiment might not high enough to have an significant effect on hematological indices.

FECAL E. COLI COUNT

E. coli is a bacterium that commonly resides in the digestive tract of both humans and animals. The change in the number of *E. coli* can be an important indicator of intestinal health. The change in the number of *E. coli* bacteria in the feces at different growing stages of chickens is shown in Table 4. The results showed that the number of *E. coli* bacteria in the feces was not affected by the herbal extracts by the age of the chicken ($p < 0.05$). In general, fecal *E. coli* count declined at 28-day-old chickens ($p < 0.05$).

Table 4: Fecal *E. coli* counts during growing stage of chickens (LogCFU/g feces).

Treatment		Day of fecal sample collection					p-value
		3	14	28	48	99	
NCTL	+n [#]	3/3	2/3	3/3	3/3	3/3	
	CFU	7.54 ^a	7.37 ^a	6.45 ^b	7.04 ^b	6.95 ^b	0.013
PCTL	+n	3/3	3/3	3/3	3/3	3/3	
	CFU	7.43 ^a	7.79 ^a	6.43 ^b	6.17 ^b	6.41 ^b	0.025
HE05	+n	3/3	3/3	3/3	3/3	3/3	
	CFU	7.88 ^a	7.83 ^a	6.83 ^b	6.59 ^b	6.75 ^b	0.041
HE10	+n	3/3	3/3	3/3	3/3	3/3	
	CFU	7.12 ^a	6.27 ^b	6.12 ^b	6.65 ^b	6.29 ^b	0.021
HE15	+n	3/3	3/3	3/3	3/3	3/3	
	CFU	7.57 ^a	7.69 ^a	7.14 ^b	7.06 ^b	6.78 ^b	0.017

[#]: Number of samples positive for *E. coli* out of the total number of samples observed; ^{ab}: Means in the same row without common letter are different at $p < 0.05$.

Several previous studies on the antimicrobial properties of *Tithonia diversifolia* have shown that aqueous extracts of *T. diversifolia* possess antimicrobial effects against the growth

of pure isolates of *S. aureus* and *E. coli* (John and Oni, 2013). Moreover, Dzinjalimala *et al.* (2023) also reported that aqueous extracts of *T. diversifolia* produced a 10 mm zone of inhibition against *E. coli*. Similarly, in a study by Liasu and Ayandele (2008), it was observed that at a concentration of 10 mg/mL, aqueous extracts of *T. diversifolia* leaves exhibited a zone of inhibition of 17 mm against *E. coli*. Herbal extracts have antibacterial effects equivalent to antibiotics under laboratory conditions (Alam *et al.*, 2009). However, when added to animal feed, higher doses are needed to demonstrate their effects (Lei *et al.*, 2018). Previous studies have also shown similar results to the results of this study, but the data have not yet provided reliable conclusions about the effectiveness of herbal extracts on the number of fecal bacteria under experimental conditions.

Table 5: Fecal *Salmonella* counts during growing stage of chickens (logCFU/g feces).

Treatment		Day of fecal sample collection					p-value
		3	14	28	48	99	
NCTL	+/n [#]	2/3	2/3	3/3	3/3	3/3	
	CFU	5.50 ^b	5.50 ^b	5.49 ^b	6.03 ^{ab}	7.16 ^a	0.021
PCTL	+/n	3/3	3/3	3/3	3/3	3/3	
	CFU	5.51 ^b	5.28 ^b	6.21 ^{ab}	5.95 ^{ab}	6.57 ^a	0.017
HE05	+/n	2/3	2/3	2/3	3/3	0/3	
	CFU	6.94 ^a	6.07 ^{ab}	5.33 ^b	5.29 ^b	0	0.042
HE10	+/n	2/3	2/3	3/3	3/3	0/3	
	CFU	6.11 ^a	5.56 ^{ab}	5.22 ^b	5.11 ^b	0	0.037
HE15	+/n	3/3	3/3	3/3	3/3	1/3	
	CFU	5.77 ^a	5.64 ^a	5.24 ^b	5.17 ^b	0	0.019

[#]: Number of samples positive for *Salmonella* out of the total number of samples observed; ^{ab}: Means in the same row without common letter are different at $p < 0.05$.

FECAL SALMONELLA COUNT

The results in Table 5 showed that the fecal *Salmonella* counts increased in the NCTL and PCTL ($p < 0.05$) and decreased in the herbal extract treatments ($p < 0.05$) following the bird age. This shows that the herbal extract has the effect of reducing the number of *Salmonella* in the period from 28 to 99 days of age. However, the fecal *Salmonella* counts didn't differ among treatments.

According to Sun *et al.* (2020), when supplementing the herbal extract, the number of *Salmonella* tends to decrease when increasing the concentration of the extract added to the chicken feed (2.64 log₁₀CFU/g in the group supplemented at 0.025% compared to 2.39 log₁₀CFU/g in the group supplemented at 0.1%) but this result does not show statistical difference. The addition of low doses of medicinal herbs has not produced clear results on harmful bacteria (*E. coli* and *Salmonella*) (Sun *et al.*, 2020). However, it has a

clear effect on increasing the number of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* (Xie *et al.*, 2018). According to Nguyen *et al.* (2015), high doses of herbal extracts treated effectively respiratory syndrome in broilers.

Table 6: Growth performance of chickens from 4 to 14 week olds in different treatments.

Item	NCTL	PCTL	HE05	HE10	HE15	SEM	p-value
Initial weight (kg)	0.30	0.34	0.30	0.31	0.30	0.01	0.136
Final weight (kg)	1.62	1.63	1.63	1.66	1.63	0.02	0.30
Average daily gain(g/d)	62.7 ^a	61.8 ^{bc}	61.0 ^c	61.6 ^{bc}	62.4 ^{ab}	0.29	0.013
Feed intake (g/d)	19.3	18.8	19.3	19.2	19.1	0.21	0.42
Feed conversion ratio	3.34	3.38	3.25	3.35	3.39	0.06	0.49

^{ab}: Means in the same row without common letter are different at $p < 0.05$; ^{SEM}: Standard Error of the Mean.

GROWTH PERFORMANCE

The effect of herbal supplements in the basal diet on growth performance was measured and presented in Table 6. The performance indices measured did not differ between treatments ($p > 0.05$), except for the average daily gain of chickens from 4 to 14 weeks old. The feed intake of chickens in NCTL was not different from those in the HE15 treatment ($p > 0.05$) but higher than other treatments ($p < 0.05$). There was no effect of herbal testing treatments on the performance of Ri crossbred chickens in a 10-week period. The present study aimed to evaluate the influence of dietary supplementation with 3 levels of HE on growth performance in chickens. However, the effect of HE on growth performance in chickens was not found. Experimental diets were formulated with popular ingredients (Table 1) and aimed to have equally levels of nutrition and energy requirement for local growing chicken (Table 2). The results of this study were similar to those reported by Sun *et al.* (2020) when adding the *Achyranthes japonica* extract to the growth parameters of chickens. It was suggested that the main mechanisms of the positive effects of herb extract on the growth performance of animals may be because of the increase in digestibility, improvement in the gut microbiome, and modification of the digestive secretion morphology (Hashemi and Davoodi, 2010). However, the exact mechanism of HE actions in improving the growth performance of broilers has not been fully understood. Therefore, further studies are needed to explain the complex effects of medicinal extracts on growth performance in chickens.

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NOVELTY STATEMENTS

The herbal extract mixture (50% *Tithonia diversifolia*, 20% *Achyranthes aspera*, 20% *Pseuderanthemum palatiferum*, and 10% *Polyscias fruticosa*) helps balance the digestive tract microflora by reducing the prevalence and occurrence of *Salmonella* bacteria in chicken

AUTHOR'S CONTRIBUTIONS

All authors contributed to the study's conception and design. Nguyen Hai Quan developed the original hypotheses, designed the experiments, and collaborated in interpreting the results; Le Duc Thao and Nguyen Van Chao collected the data for this study, conducted the statistical analyses, collaborated in the interpretation of the results; Nguyen Hai Quan, Le Duc Thao and Nguyen Van Chao collaborated in interpreting the results, and finalized the manuscript. All authors have read and approved the finalized manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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Figure 1: Photo of individual herbal extracts (Co-xuoc, da-quy, dinh-lang, and hoan-ngoc) prepared using ethanol extraction.



Figure 2: Photo of the experimental housing setup for the Ri crossbred chickens during the study period.

CONCLUSIONS AND RECOMMENDATIONS

The addition of the herbal extract mixture at up to 1.5 g/kg feed did not affect the growth performance, hematological indices, the fecal *E. coli*, but supplement of herbal extract mixture reduced fecal *Salmonella* in Ri crossbred chickens. However, it is necessary to further study the effects of this herbal extract mixture at higher levels.

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