Research Article

Evaluation of Biotic Potential of Wormwood (*Artemisia absinthium* L.) as Feed Supplement for Some Production Traits of Broiler Chicks

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Abstract | Employment of herbal plant extracts as feed additives and antibiotic alternates has been a prime focus of animal production research. This study was aimed to assess the impact of aqueous extract of perennial medicinal herb wormwood (*Artemisia absinthium* L.) on some important productive characteristics of broiler chicken including dressing percentage, feed conversion ratio, average live body weight, average weight gain and feed consumed. For the experiment, one-day old 525 unsexed Ross 308 broiler chicks (averagely weighing 42 ± 3 g per bird) were randomly provided with different concentrations of *A. absinthium* herbal extract and antibiotic tylosin, and the productive traits of chicks were determined up to five weeks. Each treatment was replicated thrice and contained 75 chicks. Treatments included T1 (control), T2 (0.25 g tylosin per liter of drinking water), T3 (0.5 g tylosin per liter of drinking water), T4 (0.25 ml of wormwood extract per liter of drinking water), T5 (0.5 ml of wormwood extract per liter of drinking water) and T6 (1.0 ml of wormwood extract per liter of drinking water). Results showed that wormwood extract (@ 1.0 ml per liter of drinking water) exhibited highest live body weight and weight gain by chicks as compared to other additives and the control treatment. Moreover, all three wormwood treatments (*i.e.* 0.25, 0.5 and 1.0 ml per liter of drinking water) caused a significant ($P < 0.01$) decrease and increase in feed consumption rate and feed conversion ratio, respectively as compared to tylosin addition. However, all additive treatments did not affect the dressing percentage, although the relative weight of internal body organs (*i.e.* liver, heart and gizzard) increased significantly ($P < 0.01$) by these treatments. It is concluded on basis of overall study results that addition of wormwood aqueous extract in drinking water had a positive impact and improved the productive characteristics of broiler chickens as compared to the antibiotic tylosin, and hence is recommended to be considered as biorational feed supplement for broiler production.

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Introduction

Poultry is one of the most economical sources of animal protein supplying a crucial portion of the humans nutritional needs around the globe. Poultry production is a fast-growing industry of agriculture as it has a significant contribution in food security (Mottet and Tempio, 2017). However, genetic improvement for acquiring maximum bird weight have exerted some negative impact on the physiology, growth and development of broiler chickens and this has forced the researchers to use antibiotics or their alternatives derived from natural medicinal plants to mitigate these negative effects (Mehdi *et al*., 2018; Oreamuno and Khalid, 2020; Ayalew *et al*., 2022).

Synthetic antibiotics are being extensively used in veterinary medicine either in lower doses to accelerate the growth and nutrition of poultry birds, to reduce the mortality and to increase the body weight of chicks, while these are given in higher doses for the prevention or treatment of various diseases. Approximately 80% of the food-producing animals receive antibiotic drugs for part or most of their rearing period (Koch *et al*., 2017; Mulchandani *et al*., 2023). Tylosin is a macrolide-group antibiotic that is used extensively in the poultry industry, particularly in broiler farming, to treat different bacterial infections. It is classified as an antibiotic being used in poultry production for several reasons, including its efficiency, low cost and few side effects. Over the years, it has remained one of the most widely used groups of antibiotics in poultry production (Alabi *et al*., 2024). Nevertheless, extensive use of synthetic antibiotics in animal production systems have resulted into many ecological and environmental consequences and human health hazards (Arsène *et al*., 2022; Islam *et al*., 2024).

Medicinal plants are one of the important sources of effective biological compounds which have been used in poultry industry since decades for their anti-inflammatory, antiparasitic and antiseptic properties (Seidavi *et al*., 2021; Ivanova *et al*., 2024). Some plant extracts stimulate the digestive system of poultry improving the body organs, particularly liver, which leads to the efficient feed consumption due to enhanced digestive enzymes production (Rai *et al*., 2003; Ivanova *et al*., 2024). Among herbal medicinal plants being used in poultry production, wormwood (*Artemisia absinthium* L.) is a promising perennial herb belonging to family Asteraceae. It is a dwarf shrub that usually grows in arid and temperate regions (Hezil *et al*., 2024). This plant contains a wide array of biologically active compounds including flavonoids, steroids, coumarins, phenolics, lipids, purines, aliphatic compounds, triterpenoids, artemisinin, artemisinic acid and dihydroartemisinic acid (Ahmad *et al*., 2023; Mashraqi *et al*., 2024). Wormwood powder or extract is being used effectively in the broiler feed as an anticoccidial agent (Kalemba *et al*., 2002). Being safe, effective and inexpensive, this herb is considered a good alternative treatment for worms and is recommended against ascariasis in laying hens (Seddiek *et al*., 2011).

The deterioration of the health and immunity of broiler chickens because of the continuous genetic improvement of some broiler strains necessitated looking for natural compounds as alternatives to synthetic antibiotics. To this end, this study was aimed to have a comparative evaluation of the biotic potential of the aqueous extract of the wormwood (Artemisia) plant and tylosin antibiotic in enhancing some productive characteristics of broiler chickens.

Materials and Methods

This 5-week experiment was carried out in a broiler rearing facility of the Department of Animal Production, Al-Mussaib Technical College, Al-Furat Al-Awsat Technical University, Iraq. A total of 525 one-day-old, unsexed chicks of the broiler breed Ross 308 (weighing an average of 42±3 g per bird) were utilized in the experiment. After being weighed, the chicks were divided into six equal treatments at random; each treatment had 75 chicks, with three replicates of 25 chicks each. The average live body weight, weight gain rate, feed consumption, feed conversion ratio, dressing percentage and weight of edible internal organs of broiler chicks were the production traits that were compared when varying amounts of Artemisia or wormwood herbal extract and the antibiotic tylosin were added to the drinking water of the broiler chicks.

During the entire experiment, feed was given in two stages: the starter stage, in which feed was used from 1 to 21 days of age, and the finisher stage from 22 to 35 days of age (marketing age). Feed was given according to the bird's requirements and needs for the nutritional compounds and elements and according to the Ross 308 broiler production guide for the year 2023 and as per National Research Council (NRC-1994) recommendations. The health and preventive program followed during the experiment included the administration of antibiotic Enrosol (10% enrofloxacin solution) against omphalitis on the first day and vaccinations against Newcastle and Gumboro diseases according to the age stages, with a mixture of vitamins given after each vaccination (Saleh *et al*., 2023).

Stems and leaves of wild wormwood plant were procured from the local market and were powdered by grinding them using an electric blender. About 250 g of wormwood powder was added in 5 liters of water and was allowed to be soaked for 5 h. After that the water was heated to 80 °C and the material was left for another 4 h to be cooled. The extract was then filtered from the impurities of the wormwood plant using a piece of muslin cloth to finally produce a pure solution of wormwood extract to be used later in the experiment. The antibiotic tylosin (500 g pack of water dissolvable powder; Interchemie Werken, Holland, The Netherlands) was procured from an authorized chemical dealer. Treatments included T1 (control), T2 (0.25 g tylosin per liter of drinking water), T3 (0.5 g tylosin per liter of drinking water), T4 (0.25 ml of wormwood extract per liter of drinking water), T5 (0.5 ml of wormwood extract per liter of drinking water) and T6 (1.0 ml of wormwood extract per liter of drinking water).

Different productive parameters of the broiler chicken including dressing percentage, feed conversion ratio, average live body weight, average weight gain and feed consumption rate were recorded at the end of each week till five consecutive weeks. The data was analyzed using the statistical program SAS (2012) to look at how different treatments affected the broiler productive traits being studied under the completely randomized design (CRD). Moreover, Duncan Multinomial Test (1955) based on the following mathematical model was used to compare the significant differences among the treatment means:

$$Yij\ (CRD) = \mu + Ti + eij$$

Results and Discussion

Impact of treatments on live body weight of chicks

On day one and during first week, there were no considerable differences between the experimental treatments in terms of average body weight trait of Ross 308 broiler chicks (Table 1). In second week, T6 (having wormwood @ 1.0 ml L^{-1} of water) had the highest but statistically insignificant average body weight as compared to other treatments. In contrast, the control treatment had the lowest average body weight. There were no significant differences between the control treatment and the second, fourth, fifth and sixth treatment except only a significant difference between the third treatment and the control treatment for the first four weeks (Table 1). Sixth treatment had the highest body weight rate in the fifth week and was clearly different from the other treatments. In contrast, the fifth and fourth treatments (both having wormwood extract) were substantially similar to each other but different from first, second and third treatments. The lowest live body weight rate was recorded in the control treatment (T1), followed by second and third ones (both having tylosin).

Table 1: *Effect of different concentrations of tylosine and wormwood aqueous extract as drinking water additives on the live body weight (mean ± standard error) of broiler chicks.*

Treatments	Average live body weight (g/bird/week)					
	One day	First week	Second week	Third week	Fourth week	Fifth week
T1 (control)	41.43±0.32A	227.40±0.52A	515.87±1.75 C	1112.83±3.74B	1830.60±4.97 B	2322.00±22.15C
T2 (0.25 g Tyl. L^{-1})	43.33±0.49A	234.67±0.20A	542.83±4.96 AB	1141.03±11.94A	1905.87±38.15AB	2350.67±17.17C
T3 (0.5 g Tyl. L^{-1})	42.83±0.42A	238.30±0.61A	526.57±4.01 ABC	1145.20±11.41A	2033.73±85.15 A	2299.00±28.87C
T4 (0.25 ml Wae. L^{-1})	43.00±0.32A	237.43±1.74A	527.77±14.65ABC	1147.10±0.40A	1950.47±2.33AB	2422.00±19.55B
T5 (0.5 ml Wae. L^{-1})	42.60±0.21A	228.77±0.38A	521.57±5.48 BC	1169.67±13.91A	1929.07±26.69AB	2476.67±15.21B
T6 (1.0 ml Wae. L^{-1})	42.76±0.18A	237.87±3.49A	549.97±6.68 A	1168.57±2.68A	1905.00±18.62AB	2599.10±12.23A
Significance	N.S.	N.S.	**	**	**	**

*Tyl. = Tylosine antibiotic, Wwe. = Wormwood aqueous extract, N.S. = Not significant, ** = Different alphabets within the same column indicate significant differences between the treatment means (Duncan's Multinomial Test @ 5% level of probability; P < 0.05).*

Table 2: *Impact of different concentrations of tylosine and wormwood aqueous extract as drinking water additives on the weight gain (mean ± standard error) of broiler chicks.*

Treatments	Average weight gain (g/bird/week)					
	First week	Second week	Third week	Fourth week	Fifth week	Total
T1 (control)	185.97±2.61B	288.47±2.75A	596.97±4.17B	717.76±7.73A	491.40±17.18C	2280.57±22.46E
T2 (0.25 g Tyl. L^{-1})	191.33±0.42AB	308.17±4.85A	598.20±16.77B	764.83±43.62A	536.97±33.51C	2399.50±16.61CD
T3 (0.5 g Tyl. L^{-1})	195.47±0.73A	288.27±3.48A	618.63±13.98AB	741.87±10.92A	515.60±3.29C	2359.83±1.25D
T4 (0.25 ml Wae. L^{-1})	194.43±1.86A	323.67±24.79A	619.33±14.95AB	742.30±3.35A	544.93±17.55C	2424.67±22.97CB
T5 (0.5 ml Wae. L^{-1})	186.17±0.26B	292.80±5.10A	648.10±19.38A	719.43±14.07A	624.90±31.26B	2471.40±32.52B
T6 (1.0 ml Wae. L^{-1})	195.10±3.67A	312.10±9.96A	618.60±9.09AB	736.43±16.12A	721.60±21.21A	2583.83±5.09A
Significance	**	N.S.	**	N.S.	**	**

*Tyl. = Tylosine antibiotic, Wwe. = Wormwood aqueous extract, N.S. = Not significant, ** = Different alphabets within the same column indicate significant differences between the treatment means (Duncan's Multinomial Test @ 5% level of probability; P < 0.05).*

Wormwood's compounds such as abrotanin and tannic acid which assist the digestive functions by increasing the gastric and intestinal secretions, may be the reason why adding wormwood to drinking water produced the best results according to the body weight table. Some previous studies have also shown that wormwood exert antibacterial, antifungal and antiparasitic effects on broiler's health (Batiha *et al*., 2020). Our results are in agreement with those of Kostadinovc *et al*. (2015) that there was an increase in the rate of live body weight and weight gain during a period of 42 days of raising broiler chickens on feed containing 15 and 20% wormwood extract as compared to the control treatment. While Çetin *et al.* (2019) showed that the addition of 23.53, 11.76 and 35.29 g/kg of wormwood extract to Ross 308 broiler feed exerted no significant differences in the average live weight and weight gain of the chicks during six weeks of the experiment as compared to the control treatment, which is contrary to what was found in current study.

Regarding tylosin's impact, our results are in contrast to the findings of Erdősi *et al*. (2015) who showed an improvement in the broiler chicks' body weight rate of broiler chicks fed with 25 and 50 ppm of tylosin per liter of drinking water as compared to the control treatment. However, Abu-Basha *et al*. (2012) reported that tylosin (@ 10 mg/kg of body weight) did not significantly affect the body weight of broiler chicks till five weeks, and these results are consistent with the findings of this study.

Effect of treatments on broiler's weight gain

Regarding weight gain, the control treatment exhibited slowest rate of weight gain for the entire duration of experiment. Till fourth week, no significant differences were observed between the other five treatments (Table 2). Overall, sixth treatment (T6), having wormwood's extract @ 1.0 ml L^{-1} of drinking water, showed maximum and significant (P < 0.01) weekly weight gain as compared to other treatments, followed by fifth treatment, fourth, second and third ones. The first treatment (control) had the lowest cumulative weight gain, while the sixth treatment (having maximum wormwood extract) had the highest weight gain with a significant difference from the other treatments.

The active phenolic compounds in the wormwood extract might have contributed to the weight gain that the broiler chicks experienced over the experimental duration. Our results are in agreement to those of Arab *et al*. (2006) and Dragan *et al*. (2010) demonstrating that supplementation of broiler feed with the extract of *Artemisia* or wormwood extract improved the feeding efficiency and daily weight gain of the Ross 308 broiler chicks. Indeed, provision of wormwood in feed can enhance the absorption of nutrients and can improve the digestion and metabolism, which leads to an improvement in the productive characteristics of flocks (Coroian *et al*., 2022; Cui *et al*., 2024). However, our results are in contrast to those of Lee *et al*. (2010) and Çetin *et al*. (2019) showing no significant effect of the dietary supplementation of broilers with wormwood meal on their performance and weight gain. This might be due to the different broiler strain (Arboracre strain male commercial broiler chicks) used in the former study.

Feed consumption rate as influenced by the treatments

The impact of experimental treatments on the broiler chicks' feed intake rate has been shown in Table 3. Statistical analysis showed no significant differences in the amount of feed consumed by the birds in second, third and fifth week. In first and fourth weeks, T1 (control) had the highest feed consumption rate significantly different ($P < 0.01$) from the other treatments, followed by tylosin fed treatments. However, T3 (with maximum tylosin) and T5 (with maximum wormwood extract) had the lowest feed consumption rate (Table 3).

The reason for lower feed consumption rate in the treatments containing wormwood extract may be attributed to the fact that it contains abundant biochemical and mineral components with high nutritional value (Lee *et al*., 2010; Ibatullin *et al*., 2022; Pierzchała-Koziec *et al*., 2024). In this way, it can be used in the management of feed consumption of broiler chickens without any harmful effects (Cetin *et al*., 2019). Our results are contradictory to those of Kostadinovic *et al*. (2015). While our results are in agreement with Lee *et al*. (2013) showing that the addition of wormwood extract to broiler feed (@ 4 to 5%) exhibited a decrease in the feed consumption rate in the first and fourth weeks of the 6-week rearing period. Moreover, Sheikh *et al*. (2020) that the use of growth-promoting antibiotics, including tylosin, has a beneficial effect on the productive performance and feed consumption of broiler chickens. While our results did not agree with what was found by Garcés-Narro *et al*. (2013), who signified that the use of tylosin in drinking water (@ 10 mg/kg of live body weight) showed a decrease in the feed consumption rate accompanied by an increase in the feed conversion ratio of the broiler chicks.

Effect of treatments on broiler's feed conversion efficacy

The feed conversion factor is related to the amount of feed consumed and the rate of weight gain. Results in Table 4 showed differential feed conversion rates by the experimental treatments. However, the differences were only significant ($P < 0.01$) for the first and fifth week as compared to the control treatment. In the first week, control (T1) and second treatment (T2)

Table 3: *Effect of different concentrations of tylosine and wormwood aqueous extract as drinking water additives on the feed consumption (mean ± standard error) of broiler chicks.*

Treatments	Feed consumption (g/ bird/ week)					
	First week	Second week	Third week	Fourth week	Fifth week	Total
T1 (control)	273.867±2.11B	476.40±4.19A	751.04±5.28A	1003.97±18.28AB	997.01±6.29A	3502.29±29.19AB
T2 (0.25 g Tyl.L^{-1})	286.24±286.24A	468.47±2.45A	751.40±8.61A	1022.29±14.80AB	1000.99±22.56A	3529.39±23.01AB
T3 (0.5 g Tyl.L^{-1})	279.55±2.03AB	472.11±8.57A	747.09±10.13A	1049.71±6.83A	1008.88±6.42A	3557.33±13.76A
T4 (0.25 ml Wae.L^{-1})	282.67±2.69A	469.09±4.74A	757.17±9.32A	978.69±9.32B	961.15±15.32A	3448.77±19.47B
T5 (0.5 ml Wae.L^{-1})	280.69±3.64AB	469.17±4.99A	760.80±13.16A	988.67±14.21B	995.57±10.15A	3494.91±34.38AB
T6 (1.0 ml Wae.L^{-1})	273.97±1.43B	477.91±3.39A	758.12±14.99A	989.25±24.18B	966.59±22.99A	3465.84±26.99B
Significance	**	N.S.	N.S.	**	N.S.	**

*Tyl. = Tylosine antibiotic, Wwe. = Wormwood aqueous extract, N.S. = Not significant, ** = Different alphabets within the same column indicate significant differences between the treatment means (Duncan's Multinomial Test @ 5% level of probability; P < 0.05).*

Table 4: *Influence of different concentrations of tylosine and wormwood aqueous extract as drinking water additives on the feed conversion ratio (mean ± standard error) of broiler chicks.*

Treatments	Feed conversion ratio					
	First week	Second week	Third week	Fourth week	Fifth week	Total
T1 (control)	1.47±0.02AB	1.64±0.01A	1.25±0.01A	1.39±0.04A	2.05±0.07A	1.56±0.02A
T2 (0.25 g Tyl.L^{-1})	1.49±0.01A	1.52±0.02A	1.26±0.05A	1.30±0.12A	1.88±0.14AB	1.49±0.02BC
T3 (0.5 g Tyl.L^{-1})	1.43±0.02BC	1.63±0.02A	1.21±0.03A	1.41±1.41A	1.95±0.02AB	1.53±0.01AB
T4 (0.25 ml Wae.L^{-1})	1.45±0.01ABC	1.62±0.08A	1.22±0.04A	1.31±0.02A	1.76±0.07BC	1.47±0.02BC
T5 (0.5 ml Wae.L^{-1})	1.42±0.02C	1.60±0.04A	1.17±0.02A	1.37±0.05A	1.59±0.16C	1.44±0.03CD
T6 (1.0 ml Wae.L^{-1})	1.40±0.02C	1.53±0.04A	1.36±0.12A	1.33±0.02A	1.34±0.01D	1.39±0.02D
Significance	**	N.S.	N.S.	N.S.	**	**

*Tyl. = Tylosine antibiotic, Wwe. = Wormwood aqueous extract, N.S. = Not significant, ** = Different alphabets within the same column indicate significant differences between the treatment means (Duncan's Multinomial Test @ 5% level of probability; P < 0.05).*

(with tylosin) exhibited maximum and significant feed conversion and treatments with wormwood extract showed minimum feed conversion ratio. In the fifth week, similar trend was observed. In general, the maximum feed conversion ratio was found in the control treatment (T1), followed by treatments with tylosin antibiotics, while wormwood extract fed chickens showed minimum feed conversion ratio. This may be attributed to the fact that wormwood contains many active substances and compounds such as volatile oils, alkaloids, flavonoids, glycosides, saponins, tannins, and coumarins which can stimulate pancreatic enzymes enhancing the synthesis of bile acids in the liver which ultimately improve the digestion and absorption process and increases the availability of nutrients being reflected in better feed conversion factor of broilers (Ekiert *et al*., 2020).

This study's results regarding feed conversion factor are in accordance to the study of Ait-Kaki *et al*. (2018) who showed best feed conversion ratio at a rate of 1.87 for broiler chickens at 42 days of age by adding 2% of wormwood powder compared to the control feed. Similarly, Roshanfekr and Mamooee (2009) showed that best growth performance was in the groups to which antibiotic AGPs (antibiotics growth promotors) were added including the antibiotic tylosin, probiotics and prebiotics. However, Shah *et al*. (2022) showed in their study that supplementation with antibiotic tylosin (@50 and 100 mg/kg of feed) for a rearing period of 42 days improved the productive characteristics of broiler chickens. A significant difference was observed between the control treatment and the two tylosin treatments in weight gain and feed conversion ratio, which are not in line with this study results.

Impact of tylosin and wormwood extract on dressing percentage and edible organs

Table 5 presents the dressing percentage and edible internal organs of broiler chicks in the experimental treatments during 5 weeks of rearing. There were no significant differences between all the studied treatments and the control treatment regarding dressing percentage. Regarding relative heart weight rate, control treatment, the fifth and sixth treatments, exhibited the lowest relative heart weight rate, which did not differ significantly from the second and fourth treatments. Regarding liver weight ratio relative to the edible internal organs, the results showed a significant superiority (P<0.01) followed by the control treatment and the second treatment than the rest treatments (Table 5). The fifth and sixth treatments (with wormwood extract) recorded the lowest relative liver weight ratio, and they did not differ significantly from the third and fourth treatments. Gizzard weight rate was found significantly lower in fourth and sixth treatments having wormwood extract supplement, while the highest values of gizzard weight rate were recorded for control and tylosin (@ 0.25 g L^{-1} of drinking water).

These results are consistent with those of Chu and Park (2022) and Marume *et al*. (2024) who showed that addition of wormwood oil had no effect on the dressing percentage of the chicks. However, our results are in contrast to Wang *et al*. (2024) who reported that addition of wormwood extract (@ 1, 3 and 5%) to the feed of broilers during the rearing period of 42 days, there was an improvement in the dressing percentage and relative weight rates of edible internal organs of the chicks.

Table 5: *Effect of different concentrations of tylosine and wormwood aqueous extract as drinking water additives on the dressing percentage and weigh of vital organs (mean ± standard error) of broiler chicks.*

Treatments	Dressing percentage	Heart (%)	Liver (%)	Gizzard (%)
T1 (control)	74.78±0.93A	0.46±0.02B	2.70±0.15A	1.62±0.16A
T2 (0.25 g Tyl.L^{-1})	76.25±0.73A	0.53±0.02AB	2.75±0.12A	1.62±0.11A
T3 (0.5 g Tyl.L^{-1})	75.75±0.38A	0.58±0.03A	2.49±0.09AB	1.37±0.04AB
T4 (0.25 ml Wae.L^{-1})	72.69±3.13A	0.54±0.03AB	2.42±0.09AB	1.25±0.02B
T5 (0.5 ml Wae.L^{-1})	74.64±0.16A	0.48±0.02B	2.23±0.12B	1.46±0.07AB
T6 (1.0 ml Wae.L^{-1})	72.70±2.18A	0.49±0.02B	2.27±0.12B	1.37±0.09B
Significance	N.S.	**	**	**

*Tyl. = Tylosine antibiotic, Wwe. = Wormwood aqueous extract, N.S. = Not significant, ** = Different alphabets within the same column indicate significant differences between the treatment means (Duncan's Multinomial Test @ 5% level of probability; P < 0.05).*

Conclusions and Recommendations

Based on overall study results, it is concluded that the supplementation of drinking water and/or feed of the broiler chicks with the aqueous extract of wormwood (*Artemisia absinthium* L.) had a positive effect on birds' growth and performance, and can enhance the productive characteristics such as live body weight and weight gain concomitantly with the reduced feed consumption, leading to an improvement in the feed conversion ratio of broiler chickens. It is recommended that the addition of 1.0 ml of aqueous extract of wormwood per liter of drinking water is the most suitable concentration to acquire desirable productive characters of broiler chicks.

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Novelty Statement

Feed supplementation is necessary to enhance broiler production. Our research indicates that wormwood (*Artemisia absinthium* L.) offers superior results, making it a recommended feed additive to improve broiler chick performance.

Author's Contribution

Ahmed Noori Al-Hussany and Mohammed Jard Kadhim: Conceived the research idea, conducted experiment, collected data and performed the statistical analysis of data, supervised the project, wrote the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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