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THE IMPACT OF SUPPLEMENTING BROILER CHICKENS WITH *TRIBULUS TERRESTRIS* POWDER ON GENE EXPRESSION OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR 1

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ABSTRACT

The *Tribulus terrestris* plant is a key component of industrial medicinal products, because it includes a high concentration of compounds with valuable biological activity. By assessing the Growth Hormone (GH) and Insulin-Like Growth Factor 1 (IGF1) gene expression in broiler chickens, this study aimed to determine the safe dosage of *T. terrestris* powder that may be added to their diet. The GC-MS data demonstrated the existence of biocompounds with a variety of pharmacological properties. *T. terrestris* contains six fatty acids: palmitic acid, stearic acid, lauric acid, arachidonic acid, tridecylic acid, pentadecylic acid, stigmasterol, and beta-sitosterol. Following the addition of plant powder to the diet at dosages of 0.5, 1, and 1.5 gm, the GH gene expression using RT-qPCR demonstrated a noticeable rise in activity. ; The highest upregulation value determined was 3.04 folds higher than the control value (0.96) at concentrations of 1.5 gm. Additionally, the data demonstrated that the liver's IGF1 expression was upregulated at 1.5 gm, increasing 1.94 times, compared to the control's (1.06). In conclusion, the study demonstrated that adding *T. terrestris* to broiler chicken diets raises the expression of the GH and IGF-1 genes. It may therefore contribute to the weight gain of broiler chicks.

Keywords: *Tribulus terrestris*, Growth hormone, IGF1, Ross308

INTRODUCTION

Worldwide, poultry is the most produced animal and offers a cheap source of protein in a quick manufacturing process. In chicken farming, feed additives are crucial to maintaining these high output levels. As a result, they are frequently employed to boost output and stop the easy spread of illnesses. (Gržinić *et al.*, 2023).

Modern broilers are typically fast-growing birds marketed at 5-6 weeks of age. (Ayodele *et al.*, 2023). Since feed costs are the primary determinant of a nations, regions, or chicken company's competitive-ness, the poultry industry depends on balanced nutrition and competent health

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management. Several considerations including worldwide supply of feed materials and local tariff costs, among other factors, affect the cost of feed (Janocha *et al.*, 2022). Feed additives are now essential for optimal performance and productivity in modern poultry production. The availability of high-quality, yet less expensive feed alternatives have a significant impact on the level of development and economy in poultry farming. It remains a priority under the program "Development of agriculture and organization of agricultural products, raw materials, and foodstuffs" (El-Baz and Khidr, 2024). One of the primary objectives of the country's agri-industrial community is to supply the population with high-quality food. Competitive agricultural products and long-term production expansion are the only ways to address the related problems. (Tabashsum *et al.*, 2023).

Growth hormone (GH) is a 22-kDa single-chain polypeptide hormone produced and secreted by the anterior pituitary. The GH gene sequences in many poultry breeds are highly homologous, albeit less so than in mammals (Mo *et al.*,2022). Except for the pituitary gland, GH is expressed in many tissues, such as the thymus gland, spleen, ovary, liver, kidney and lymphocytes. Apart from the endocrine mechanism, tissues and cells can also operate in an autocrine or paracrine manner. GH stimulates bone and muscular growth, protein synthesis and fat breakdown in animals. Furthermore, GH controls gender differentiation, sexual maturation, pregnancy, lactation, and reproduction (Rotwein and Chia, 2010).

The IGF1 gene is one of the most crucial genes, since it corresponds with a wide range of developmental and productive activities in chickens (Mohammed *et al.*, 2017 and Hosnedlova *et al.*, 2020). The IGF1 gene has also been shown to alter growth rate, body composition, and lipid metabolism in chickens. Furthermore, the IGF1 gene has a critical function in cell growth, proliferation and differentiation (Ipsa *et al.*, 2019 and El-Attrouny *et al.*, 2020). As the chicken ages, the plasma concentration of the IGF1 rises. It is worth noting that IGF1 is located in the liver, as well as other tissues, including muscle, kidney and brain (Jia *et al.*, 2018). *T. terrestris* L. is a Mediterranean herb belonging to the Zygophyllaceae family. Its fruit has diuretic, aphrodisiac, galacta-gogue, general and uterine tonic properties. The fruit's main ingredients are alkaloids (harman, tribulusamides, norharman). Other components include steroidal saponins such as protodioscin, gitonin, and tribulosaponins A, B and flavonoids (quercetin, kaempferol, rutin) (Končić 2017).

The purpose of the present study was to evaluate the powder of the fruits of the *T. terrestris* plant, which grows naturally in Iraq, as a feed supplement. The current study also aims to determine many significant chemical compounds using gas chromatography-mass spectrometry (GC-MS) and to investigate the expression of the GH and IGF1 genes.

MATERIALS AND METHODS

Plant collection and classification

The plant was collected from Nineveh Governorate, where the fruits of the *Tribulus terrestris* plant were gathered between November and December-2023). After being cleaned to get rid of the dust, the plant was dried away while being constantly stirred to keep it from growing mold and to protect the compounds it contained. The dried fruits were then ground using an electric grinder to obtain a fine powder, and they were stored in opaque glass boxes until use.

Preparation of plant extracts

The process was carried out using the continuous extraction method (Soxhlet) (Al-Assaf *et al.*, 2023) utilizing organic solvents hexane, ethyl acetate and ethyl alcohol. Figure (1A), 100 gm of plant powder under the study were weighed,

inserted in a thimble made of cellulose, placed inside a Soxhlet device containing 1000 ml of solvent, and left for 24 hrs. To complete the soaking process, and to provide more chance for extracting the active substance in the sample, the mental heater was turned on to begin the extraction process. It took 48-72 hrs until the color of the solvent inside the extraction unit turned transparent, indicating the end of the extraction process. The extracts were concentrated using a rotary vacuum evaporator at a temperature ranging between (45-50°C) until a thick liquid of 30 ml was obtained. It was stored in the refrigerator at 4°C in bottles with a tight, dark-colored cap until use.

Figure (1A): Soxhlet device, (1B) GCMS-QP2010.

Analysis of *T. terrestris* hexane extract by GC-MS

The hexane extract was analyzed using a GC-MS (GCMS-QP2010 Ultra, Shimadzu Co., Japan) (Figure 1B). The device is located in the food sciences department, agriculture college, Basrah University. The device was supplied with a 0.25mm film thickness, a 0.25 mm inner diameter and a 30 m length capillary column Rtx-5. The experiment was carried out in electron impact mode with an ionization voltage of 70 eV. The injector and the detector temperatures were set at 250°C and 280°C, respectively. At a flow rate of 1.2 ml/min was used with 99.9% pure helium served as the carrier gas. For analysis, approximately 1 ml of the material was injected. The oven temperature was programmed to rise from an initial temperature of 35°C, kept for 3 minutes, to 240°C at a rate of 5°C/min, then to 280°C at a rate of 3°C/min with a hold time of 4 minutes. The identification of compounds was based on the analysis of mass spectral data using NIST and WILEY libraries as references (Zribi *et al.*, 2019).

This study was conducted in the animal house at the College of Veterinary Medicine, University of Mosul for 42 days.

Chicks Source

In this study, 120 one-day old broiler chicks (Ross 308) with an average weight of 44 gm were purchased from the supplier Nibras Company in Nineveh, Mosul Governorate.

Experimental design

The experiment included four treatments, each with three replicates (10 chicks/ replicate). The replicates were distributed randomly and kept under a temperature of 34°C for 14 days. The temperature then reduced to 28°C and continued until the end of the experiment. The birds were kept for 42 days and were given feed and water ad libtium throughout the experimental period. The treated groups were fed diets containing different levels of *T. terrestris* powder as follows.

* Control treatment (no *T. terrestris* was added); treatments (1^{st}, 2^{nd} and 3^{rd}) were provided with the *T. terrestris* at a concentration of 0.5 g/kg, 1 g/kg and 1.5 g/kg, respectively.

RNA extraction

Liver samples were used for general RNA extraction using a ribosomal RNA extraction kit (Genome, USA) according to the instruction provided. The primer is designed according to (Anh *et al.*, 2015).

Table 1: Ingredients and nutrient contents of basal diet in the experiment.

Feed materials	starter	Finisher
Maize	30	69.5
Wheat	27	-
Soybean meal	29	19.5
Animal protein concentrate	10	10
Soybean oil	3	-
Limestone	0.7	0.7
Iodized salt	0.3	0.3
Protein percentage	22.815	18.69
Represented energy	3044.1	2993

*Al-Hayat Company/Jordanian origin. Contains ٢٢% protein, 2800 kilocalories, 12% fat, 25% ash, 5% calcium, 2.9% phosphorus, 2.55% methionine + cysteine, 2.8% lysine. The chemical composition was calculated according to the analyses of feed materials mentioned in the NRC (1994).

Reverse transcription

was carried out using a special kit (Add Script RT master 2X, Korea) according to manufacturer's instructions

The final volume used was 35 µl, and these tubes were placed into a thermocycler device.

The thermocycling was as follows; at 25°C for 10 mins. priming, reverse transcription at 50°C for 60 mins. and inactivation at 80°C for 5 mins.

The cDNA samples were kept at -80 °C for the purpose of being used in gene expression.

Table 2: Sequences of the primer pairs used in gene expression.

No. Primer	Name Primer	Sequence 5' – 3'	amplicon size (bp)
1.	cGH-F	TCCCAGGCTGCGTTTTGTTACTC	429
2.	cGH-R	ACGGGGGTGAGCCAGGACTG	429
3.	IGF-I-F	TCAAGAGAAGCCCTTCAAGC	813
4.	IGF-I-R	CATTGCGCAGGCTCTATCTG	813

Gene expression detection using RT-PCR

The gene expression of the studied genes was detected using reverse transcription-polymerase chain reaction (RT-PCR) using a US Applied Biosystem Step One instrument, and the data was analyzed using Step One Software V. 2 software. The reaction solution included the addition of Add SYBER Master mix (2X concentration) of Korean origin to the strep PCR tubes to prepare the reaction solution in a volume of 20 microliters as follows:

10 µl SYBER master MIX
1 µl of reverse primer (20 pmol)
1 µl of forward primer (20 pmol.)
2 µl of cDNA (template/sample)
6 µl nuclease-free water

The operation of the device is programmed according to the instructions included with the RT kit, and is as follows:

1. Stage I Initial denaturation 95°C 10 minutes
2. Stage II PCR cycling for 45 cycles
a.Step I denaturation 95°C 30 seconds
b. Step II Annealing 62°C 30 seconds
c. Step III extension 72°C 60 seconds
3. Stage IV post PCR hold stage 25°C ∞

Statistical analysis

Statistical analyses were performed via IBM SPSS Statistics 26. (SPSS In. Chicago, IL., USA). One-way analysis of variance (ANOVA) has been used to evaluate quantitative data.

RESULTS

The results of the GC-MS analysis of the *T. terrestris* plant showed the presence of many biocompounds including eight fatty acids. Table (3), the area under the curve was 5.14% for palmitic acid (omega 7)). Forstearic acid, it was 15.34%; and it is also an important fatty acid found in animal cells and vegetable fats. For arachidonic acid and

lauric acid, it was 1.03% and 0.93%, respectively. It was 0.89%, for Gamma-Tocopherol which is one of the main forms of Vitamin E.

The area under the curve for the two compounds, Stigmasterol and β-sitosterol, was 1.45% and 13.71%, respectively.

Table 3: Retention time and name of phytochemical constituents identified in the hexane extracts of *T. terrestris* using gas chromatography-mass spectrometry**.**

Number	Curve number	Retention time (min)	The area under the curve	% of the area under the curve
1	Palmitic acid	18.132	3022452	6.43
2	Stearic acid	21.165	7214945	15.34
3	Arachidonic acid	25.407	486119	1.03
4	Lauric acid	28.705	436947	0.93
5	Gamma- Tocopherol	25.342	3182238	0.89
6	Pentadecylic acid	16.627	449572	0.96
7	Stigmasterol	32.869	683993	1.45
8	beta-Sitosterol	34.602	6446228	13.71

Gene expression of GH and the IGF1 hormone using RT-qPCR

The present study showed that adding *T. terrestris* plant fruit with concentrations of 0.5 gm, 1 gm and 1.5 gm to the diet up-regulated GH expression compared to the control. The highest value was at concentrations of 1gm and 1.5 gm, reaching 2.32 and 3.04 folds compared to the control (0.96) (Figure 2).

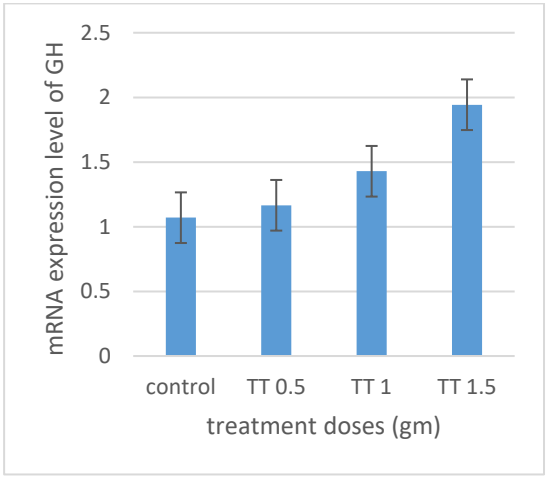

Figure 2: The expression of the gene responsible for producing the GH hormone in Ross 308 chickens was measured using RT-qPCR after adding *T. terrestris* fruit powder

After adding the *T. terrestris* plant fruit powder at concentrations of 0.5 gm, 1 gm and 1.5 gm to the diet, there was a noticeable increase in the gene expression activity of IGF 1, as the highest value was at concentrations of 1gm and 1.5 gm, reaching 1.42 and 1.94 folds changes, respectively, compared to the control (1.06).

Our findings also showed that adding 0.5 gm, 1gm and 1.5 gm/kg Feed of *T. terrestris* increased broiler growth dramatically over a period of 42 days Table (4) and Figure (4).

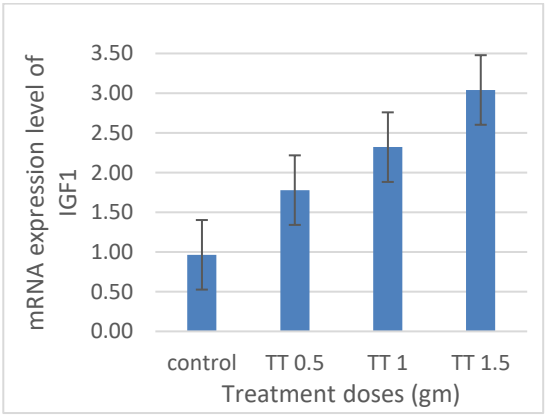

Figure 3: The expression of the gene responsible for producing the IGF-1 hormone in Ross-308 chickens was measured using RT-qPCR after adding *T. terrestris* fruit powder.

Table 4: Live weight of broilers chickens after adding *T. terrestris* fruit powder

Treatments	Control	TT 0.5 g/kg	TT 1 g/kg	TT1.5 g/kg
Live weight	d 2.94±2593.88	c 2.85±2757.63	b 4.78±3126.21	a 5.69±4147.58

Horizontally different letters indicate significant differences between the coefficients at a probability level of 0.05 ($P \geq 0.05$).

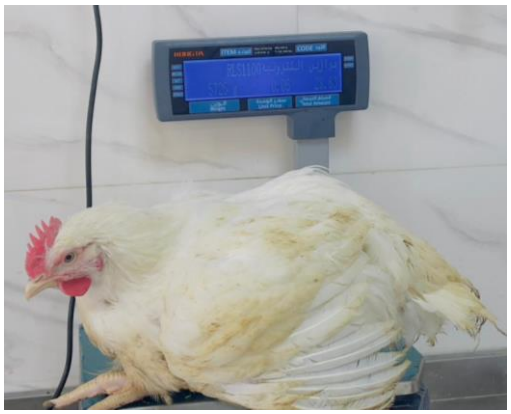

Figure 4: Broiler chickens Ross-308 fed 1.5 gm/kg *T. terrestris* powder was added to the diet at 42 days old, weighing 5.725 kg.

DISSCUSION

The most prevalent fatty acids in the body are saturated fatty acids, which are produced internally from amino acids and carbohydrates or obtained from diet. They play a crucial role in the development of cell membrane. (Carta *et al.*, 2017 and Avato and Tava, 2022).

The three fatty acids Palmitic acid, Lauric acid, and Oleic acid are among the essential fatty acids found in plants. They are produced in large quantities and belong to the omega 3, 6 and 7 acids group. Furthermore, the primary unsaturated and saturated oils include palmitic acid, lauric acid, and oleic acid; they have anti-inflammatory and metabolic-boosting qualities. (Buckley *et al.*, 2017 and Tkachenko *et al.*, 2020, Saeed *et al.*, 2024).

One plant is thought to be high in stearic acid is coconut butter, which is used extensively in the cosmetics industry to soften plastics and harden soap. (Kenar *et al.*, 2017).

An essential omega-6 polyunsaturated fatty acid, arachidonic acid (AA; 5,8,11,14-cis-eicosatetraenoic acid) regulates membrane fluidity and other bodily metabolic functions. It serves as a direct precursor to physiologically active prostaglandins (series 2), leukotrienes, and a variety of eicosanoids (Dedyukhina *et al.*, 2014 and Shanab *et al.*, 2018). Animals and humans cannot produce this fatty acid, and it must be supplemented in theirdiets.

Each tocopherol contains a chromanol ring and a 16-carbon phytyl chain. Tocopherols are classified as α, β, δ, and γ based on their methyl group number and position on the chromanol ring (Donnelly *et al.*, 2022). They are natural antioxidants that prevent lipid oxidation in biological systems by stabilizing hydroperoxyl and other free radicals. The radicals and antioxidants in tocopherol stabilize the oil (Padhi *et al.*, 2017).

They are compounds with important pharmacological activities. Therefore, the fruits of the *T. terrestris* plants have been used in traditional medicine as antimicrobial, anticancer, diuretic, anti-inflammatory, antioxidant, anti-asthma, and protective activities. They are rich in δ-Tocopherol, α-Tocopherol, Vitamin D, Vitamin K, Ergosterol, β-Sitosterol, Stigmasterol, and a large group of fatty acids such as palmitic acid and linolenic acid (Zhu *et al.*, 2017 and Khalid *et al.*, 2022). When T. terrestris powder was introduced to a basal diet at 0.75 g/kg between the ages of 1 and 35 days, it had no effect on the growth (feed intake, weight increase, and feed conversion ratio) of the hens (Al-Garadi *et al.*, 2022).

The positive effects of functional plant extracts on disease prevention and broiler chicken performance are being investigated with increasing attention. *T. terrestris* is one of the herbal medicinal plants, and its extract is now employed in pharmaceutical industries, as well as the creation of nutritional supplements and functional foods (Abbas *et al.,* 2024). In addition, recent pharmacological studies have extensively described the main active and bioactive compounds, which support the biological effects of *T. terrestris* (Saeed *et al*., 2024).

According to earlier research, including plants in broiler diets improved body gains and feeding efficiency. They are also suggested as a substitute for antibiotics and growth stimulants in broiler feed. (Lee *et al.*, 2023 and Oso *et al.*, 2019). *T. terrestris* contributes to broiler growth in two ways. It enhances digestion by lengthening and widening the ileal villi, which increases the absorption surface area, expression of brush border enzymes, nutrient transport systems, and body weight. The other may be related to its active ingredients, particularly steroid glycosides (saponins), glycosides, protodioscin, flavonoids, and alkaloids. (Saxena *et al.,* 2009 and Al-Akash *et al.,* 2022).

As an alternative to adding antibiotics to feed, to boost growth, the addition of plants has recently drawn more attention. The primary mechanism of action of growth-promoting feed additives is the modulation of the gut microbiota, thereby improving the microbial environment and controlling potential infections. This is particularly pertinent during crucial stages of the animal production cycle, in the context of the environment, or when there are sanitary disturbances. (Al-Baadani *et al.,* 2023).
Growth hormone (GH) and insulin-like growth factor-I (IGF-I) are two examples of somatotropic axis genes that improve chicken growth performance and carcass quality. (Windisch *et al.,* 2008 and Dong *et al.*, 2024). The somatotropic axis genes play critical roles in the genetic regulation of growth performance, and they have growth-promoting effects on almost every cell in the body, including skeletal muscle, cartilage, bone, liver, kidney, nerves, skin, hematopoietic cells, and lungs. The molecular genetic mechanism by which they stimulate growth and carcass quality traits in chicken populations is poorly understood (Jia *et al.,* 2018 and Sinpru *et al.,* 2021). Therefore, the current study evaluated gene expression of GH in plasma and IGF-1 in the treated liver.

Our findings revealed enhancement in growth performance, including body weight in response to adding *T. terrestris* plant from the first week of broiler chicken life, especially at 1.5 g/kg concentration.

Other research groups have reported similar effects. This is especially significant considering that skeletal muscles constitute approximately 50% of the total body weight in chickens, playing a vital role in determining edible meat yield, as well as overall livestock production (Righi *et al.,* 2018). Avian IGFs' physiological mechanisms of action differ slightly from mammals; chickens have large levels of IGFs in free form. Several studies showed that exogenous IGF treatment has an impact on growth rate and body fat loss (Dong *et al.,* 2024).

IGF promotes cell replication, DNA, RNA, and protein synthesis, as well as ornithine decarboxylase activity. IGF affects mesodermal cell differentiation in several ways, where rat osteoblast precursors, muscle cell progenitors, and mouse erythroid cells differentiate. (Hosnedlova *et al.,* 2020). In vivo studies suggested that IGF-1 infusion is more effective in malnourished animals than in healthy ones, and treatment does not increase muscle protein synthesis in well-fed mammals and birds (Ipcak and Alcicek, 2018 and Iresjö *et al.,* 2022).

CONCLUSIONS

According to the results, broiler chicken diets containing *T. terrestris* plant increased the expression of the GH and IGF-1 genes. Consequently, it may contribute to the weight gain of broiler chickens. Overall, the results of this study demonstrated that using T. terrestris plants as herbal supplements can substitute antibiotics that promote growth without causing any negative effects.

CONFLICT OF INTEREST

The author reports no conflicts of interest and is responsible for the content and writing of the paper.

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تأثير إضافة مسحوق نبات *T. terrestris* كمكمل غذائي مستدام على التعبير الجيني لهرمونات GH وIGF1 في دجاج اللحم

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يحتوي نبات *T. terrestris* على نسبة عالية من المواد الكيميائية ذات النشاط البيولوجي، مما يجعله عنصرا هاما في المنتجات الطبية الصناعية. ومن ناحية أخرى، فإن الأبحاث المتعلقة بالدجاج اللاحم محدودة. لذلك كان الغرض من هذه الدراسة هو تحديد الكمية الآمنة من مسحوق *T. terrestris* التي يمكن الاستفادة منها في علف الدجاج اللاحم من خلال قياس أداء النمو. وأظهرت نتائج GC-MS وجود مركبات حيوية تمتلك مجموعة متنوعة من الأنشطة الدوائية في *T. terrestris* تحتوي على ثمانية أحماض دهنية: palmitic acid, lauric acid, stearic acid, arachidonic acid, tridecylic acid, pentadecylic acid, stigmasterol, beta-sitosterol. أظهر تعبير هرمون GH باستخدام RT-qPCR في بلازما دجاج روس-٣٠٨ بعد إضافة مسحوق النبات بتراكيز ٠,٥ و ١ و ١,٥ جرام إلى العليقة زيادة ملحوظة في نشاط التعبير الجيني GH مقارنة بالمجموعة الضابطة. وكانت أعلى قيمة عند التراكيز ١,٥ غم حيث بلغت ٣,٠٤ أضعاف مقارنة بالضابطة التي بلغت ٠,٩٦، في حين بلغ تعبير هرمون IGF1 في الكبد عند ١,٥ غرام ١,٩٤ أضعاف مقارنة بالضابطة التي بلغت ١,٠٦. ويمكن الاستنتاج أن إضافة مسحوق ثمار نبات T. terrestris إلى الوجبات الغذائية للدجاج اللاحم يرفع التعبير الجيني لهرمون النمو وعامل النمو IGF-1. وبالتالي قد يسهم في زيادة وزن دجاج اللحم.