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Evaluation the sustained effect of *Morus nigra* leaves on growth hormones and insulin-like factor in broiler chickens

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A B S T R A C T

Morus nigra contains a high concentration of chemicals with biological activity, making this plant as an important element in industrial medical products. However, it is still limited information about *M. nigra* role in broiler chickens. Therefore, the purpose of this study was to determine the safe amount of *M. nigra* powder that can be utilized in the feed of broiler chickens by measuring growth performance. The results of GC-MS showed the presence of bio-compounds possess variety of pharmacological activities in *M. nigra* . It contains six fatty acids Phytol, palmitic acid, stearic acid, arachidonic acid, lauric acid and Squalene. The effect of plant powder at concentrations of 1, 2 and 3 g to the diet was examined in the plasma of Ross 308 chickens using RT-qPCR. Findings showed a noticeable increase in the activity of Growth hormone expression compared to the control. The highest value was at concentrations of 3 g reaching 2.75 fold changes, compared to the control, which was 0.96. In contrast, the expression of the IGF1 hormone in the liver at3g reaching 2.66 fold changes, compared to the control 1.02.

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Introduction:

Poultry production is one of the most important livestock sectors due to its cheapest price as a source for animal proteins, including eggs and meat that normally, use in human consumption. The cost of food represents between 60-75% of the total cost of poultry production. However, the availability of feed is decreasing as a result of the increasing requirements of population. Therefore, finding economical, cost-effective and locally available alternatives to traditional feed sources can be one way to reduce poultry feeding costs [1][2]. Poultry meat is widely used as a source of animal protein in human growth and development and it has it's a high nutritional quality, delicious taste, and low cost [3].It has been shown that adding mulberry leaves t the list of nutritional can change the structure of poultry feed, high nutritional value, to the list of nutritional alternatives for poultry feed. Mulberry leaf feed can be used successfully in the diet of poultry because it is easily digested (70-90%) by herbivores as well as monogastrics and they are found to contain little or no anti-nutritional factor or toxic compounds [4]. It positively affects the growth performance and nutrient utilization of broiler chickens. It has been found that mulberry leaves contain a variety of active substances, including polysaccharides, flavonoids, and polyphenols, which can reduce blood glucose and triglycerides, and have the function of antioxidant and regulation of fat metabolism. The yolk color improves in eggs of birds fed raspberry leaf powder, which could be due to excessive amounts of xanthophylls in the leaves [5]. It positively affects the growth performance and nutrient utilization of broiler chickens. Mulberry leaves also have huge potential effect to reduce feed cost as they can be used in place of expensive protein sources such as soybean meal and fish meal in poultry diets.

Mulberry leaves also have huge potential to reduce feed cost as they can be used in place of expensive protein sources such as soybean meal and fish meal in poultry diets[2]. The study aims to find out whether it is possible to add mulberry leaves up to certain limits in poultry feed and to know its effect on performance.

Materials and Methods

Plant collection and classification

The plant was collected from Nineveh Governorate, where the leaves of the *Morus nigra* plant were collected during the period between the May and June. The plant was then cleaned of dust and dried away from external influences (sunlight, dust, humidity) with continuous stirring to prevent it from becoming moldy and to preserve the compounds it contains. This is to avoid the effect of the enzymes present in the plant. The dry fruits were then ground using an electric grinder to obtain a fine powder, then they were stored in opaque glass boxes until use.

Analysis of *M. nigra* hexane extract by GC-MS

The hexane extract was subjected to GC-MS analysis using the GCMS-QP2010 Ultra instrument, manufactured by Shimadzu Co., Japan figure (1B). Found in the food sciences department, agriculture college, Basrah university. The device was outfitted with a 0.25 m film thickness, 0.25 mm inner diameter, and 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 detector temperatures were set at 250°C and 280°C, respectively. At a flow rate of 1.2 mL/min, 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 the NIST and WILEY libraries as references [7][8].

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

The Experiment Treatments

This study was conducted in the animal house of the College of Veterinary Medicine / University of Mosul, where (144) chicks Ross-308 one-day old were prepared from the hatcheries of Nineveh Governorate. The duration of the experiment was (42) days. The chicks were divided into four groups and each group was divided into It is divided into three replicates, and each replicate contains (12) chicks. The hall is of the open type and ground-rearing, and is divided into 12 cages with dimensions (2.5 * 1.5 m**).**

As it had all the appropriate environmental conditions for raising birds, including temperatures, lighting, and ventilation, the hall was equipped with plastic manholes, feeding troughs, and an air vacuum. Dettol was used to sterilize the hall, and after a day it was sterilized with Virokil, then 5 cm thick sawdust was used as a floor mat [9].

Feeding: The starter diet was fed from the age of 1-28 days, and the Finisher diet from the age of 28-42 days Table (1). The treatments were divided as follows:
1-The first treatment: fed the standard diet without any additives (control).
2-The second treatment: fed the standard diet and adding (1 g/kg feed)
3-The third treatment: fed the standard diet and adding (2 g/kg feed)
4-Fourth treatment: fed the standard diet and added (3 g/kg feed)

Table 1. Components of the prefix and final relations 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 44% 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 analyzes of feed materials mentioned in the NRC (1994) [10].

RNA extraction: According to the attached instructions and as follows: Kit Genome, USA). Liver samples stored at -80°C were used for general RNA extraction using a ribosomal RNA extraction kit.
RNA isolation and extraction were performed in a well-sterilized cabinet, using 100 mg of liver and 1 ml of tissue lysis solution (RL) triturated in a sterile ceramic mortar. The mixture was crushed well before being inserted in special tubes from the isolation and extraction kit. The sample was left for five minutes. To achieve full disintegration of the nucleoprotein complex, use a temperature of 25°C. The tubes were filled with 200 µl chloroform and vortexed for 15 seconds before being kept at room temperature for 3 minutes.
The samples were then put in a cooled centrifuge set to 12,000/min for 10 minutes at 4°C. After extracting the sample from the centrifuge, the mixture is divided into three phases: the yellow phenol-chloroform phase, the medium phase, and a colorless, watery upper phase containing RNA. In this stage, the Rnase-free microcentrifuge tube phase was transferred to fresh tubes.
Add 0.5 liter absolute ethanol. Mix thoroughly. It was discovered that precipitates developed shortly after adding the ethanol. The mixture was then transferred to the spin column with filter and centrifuged at 12,000 rpm for 30 seconds at 4 °C.
After discarding the debris from the tube, add 500µl of RPI wash buffer solution. The tubes were centrifuged at 12,000 per minute for 30 seconds. After discarding the sediment from the bottom of the tubes, add 500µl of the wash buffer RW solution and leave at temperature. After a minute, the sample was centrifuged at 12,000/min for 30 seconds before being discarded; this process was repeated. Place the column in the centrifuge for two minutes at a speed of 12000/min.
The dried column was put inside a 1.5 ml tube, and 100-50 µl of Rnase-free water was poured directly to the membrane holding the RNA and maintained at room temperature for two minutes. The tubes were then placed in a centrifuge at a speed of 12,000/min for two minutes in. This procedure yielded pure RNA, and samples were kept at -80°C until required.

The process of copying RNA to cDNA (reverse transcription)

In this phase, the RNA was converted using a specific kit, namely Add Script RT master (2X concentration) of Korean origin, according to the reaction solution employed in line with the manufacturer's instructions as shown in Table 2.
1.10µl AddScript RT master.
2.20 µl RNA.
3.5 µL nuclease-free water
1 µL Forward Primer
1 µL Reverse Primer
The final volume was 35 µl, and these tubes were placed for the polymerase chain reaction device (PCR) with a volume of 200 µl. The PCR tubes were transferred to a thermal cycler to convert and copy the RNA into cDNA according to the following organization
1.Priming 25°C 10 minutes
2.Reverse transcription 50°C 60 minutes
3.RT inactivation 80°C 5 minutes
4.Hold 12°C∞
After completing the transcription process, the cDNA samples were kept at -80 C for the purpose of being used in gene expression[11].

Table 2. Information for primers sequence

No	Name Primer	Sequence (5' – 3')	Length bp
1.	cGH-F	TCCCAGGCTGCGTTTT GTTACTC	429
2.	cGH-R	ACGGGGGTGAGCCAG GACTG	429
3.	IGF-I-F	TCAAGAGAAGCCCTT CAAGC	813
4.	IGF-I-R	CATTGCGCAGGCTCT ATCTG	813

Results and discussions:

GC-MS analysis revealed that the compounds present in the hexane extract of mulberry leaves are the presence of many fatty acids at different

concentrations Table (3) and Figures (1),(2),(3),(4),(5)and (6).

Phytol was the main ingredient identified with an under the curve of 43.69% and is an acyclic diterpene with anti-cancer, anthelmintic, anti-hepatic, anti-cholesterolemic, anti-androgenic, anti-microbial, antioxidant, anti-arthritic, and glucose regulation activities and it is an immune stimulant [12][13]. Followed by Palmitic acid with an under the curve of 12.17%**.** This fatty acid is known to perform multiple essential biological functions at the cellular and tissue levels [14].

The results showed the presence of the fatty acid Squalene with an under the curve of 2.24%. Squalene can be found in some fish oils, especially shark liver oil, in large quantities and in some vegetable oils in relatively smaller quantities. Many studies have shown results proving some of the vital activities of Squalene such as anti-cancer, antioxidant, drug carrier, detoxifying, and skin moisturizing activities. According to promising results from recent studies, squalene is considered an important substance in practical and clinical uses with enormous potential in the food and pharmaceutical industries [15] [16][17].

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

No	Curve name	retention time (min)	area under curve	%area under curve
1	Phytol	21.662	17329683	43.69
2	Palmitic acid	18.134	4826007	12.17
3	Stearic acid	21.231	5562823	14.02
4	Arachidonic acid	25.407	947541	2.39
5	Lauric acid	28.705	861819	2.17
6	Squalene	33.179	889502	2.24

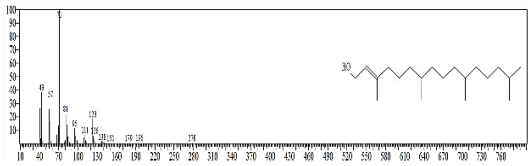

Figure 1. The curve and the structural formula of the phytol

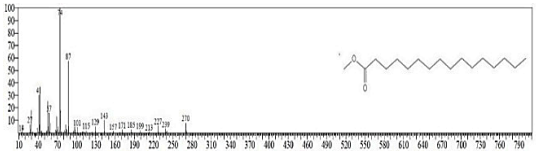

Figure 2. The curve and the structural formula of the Palmitic acid

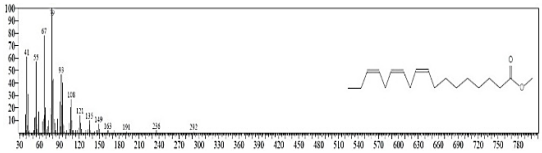

Figure 3. The curve and the structural formula of the Strearic acid

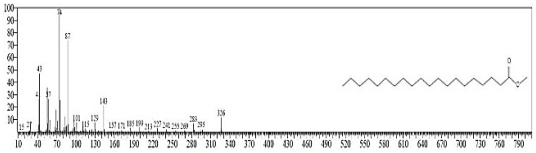

Figure 4. The curve and the structural formula of the Arachidonic acid

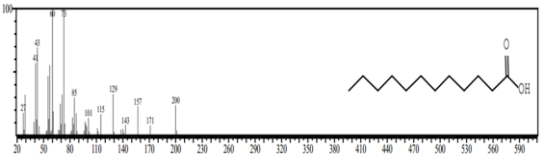

Figure 5. The curve and the structural formula of the Lauric acid

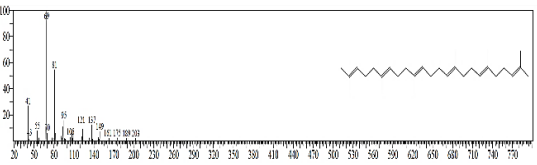

Figure 6. The curve and the structural formula of the Squalene

The results in Figure (7) showed measurement of GH expression using RT-qPCR in the liver of Ross 308 chickens. After adding *M. nigra* leaves powder at concentrations of 1, 2, and 3 g to the diet, a significant increase in GH gene expression activity was observed compared to the control group. The female officer who consumed the bush. standard only, as the highest value was at the concentrations of 2 and 3 g, where the changes reached 2.22 and 2.75 fold, respectively, compared to the control group, which amounted to 0.96 fold.

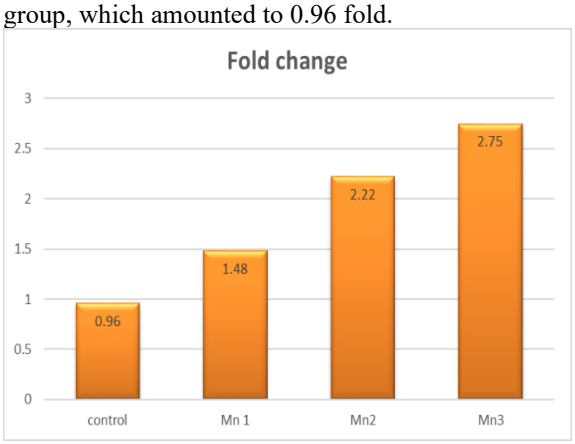

Figure 7. Measuring the expression of the gene responsible for the production of growth hormone in Ross 308 chickens using RT-qPCR after adding *M. nigra* leaf powder.

Expression of the gene responsible for IGF1 production using *M. nigra* plant RT-qPCR

The results in Figure (8) showed measurement of gene expression of the gene responsible for the IGF1 hormone using RT-qPCR in the liver of Ross 308 chickens. After adding *M. nigra* leaf powder at concentrations of 1, 2, and 3 g to the diet, a noticeable increase in gene expression activity was observed. For IGF 1 compared to the control group

that consumed the standard diet only, the value was highest at concentrations of 2 and 3 g, reaching 2.16 and 2.66 fold changes, respectively, compared to the control group, which reached 1.02 fold.

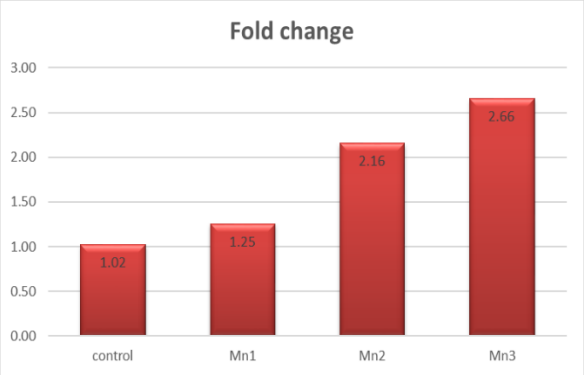

Figure 8. Measuring the expression of the gene responsible for producing the IGF-1 hormone in Ross 308 chickens using RT-qPCR after adding *M. nigra* leaf powder.

Mulberry leaves are rich in nutrients and bioactive compounds, which can be used as cost-effective feed materials for livestock and poultry [18] .

In the current study, the use of *M. nigra* leaf powder at rates of 1, 2, and 3 g/kg diet increased the growth performance of broiler chickens over a period of 42 days. Previous studies reported

Fermented mulberry leaf powder can increase breast muscle productivity and improve the performance of broiler chickens [19]. The flavonoids in mulberry leaves, including rutin, isoquercitrin, kaempferol-3-(6-rhamnosyl glucoside), and quercetin 3-(6-malonyl glucoside), are the main active substances of mulberry leaves [20]. Mulberry leaves have been shown to have multiple biological effects, such as antioxidants, immune regulation and strengthening Growth [21].

Previous studies have found that nutritional supplements containing mulberry leaves can improve liver fat metabolism and ovarian function in aged breeder chickens, thus increasing egg-laying performance [22].

The addition of dried seaweed aqueous extracts can improve feed efficiency, digestibility, and serum IgG levels, and reduce the density of potential enteric pathogens.

The beneficial effects on performance are partly due to improved digestion and general health conditions of the animals due to improvement in antioxidant status or changes in gene expression of intestinal immunity, barrier function genes, and liver growth hormone receptor genes in broilers [23].

Growth hormone (GH) is synthesized and released from the anterior pituitary gland into the bloodstream, thus stimulating the liver to produce IGF-1, which is the primary mediator of the effects of GH. IGF-1 stimulates the body's systemic growth and has growth-promoting effects. In addition to insulin, it can IGF-1 also regulates cell growth and development, especially in neurons, as well as cellular DNA synthesis [24].

Hosseini et al., 2016 [25] noted that the use of four medicinal plants (*Salvia officinalis*, *Matricaria chamomilla*, *Teucrium polium* and *Origanum majorana*) added to the diet causes broiler chickens to improve their immune system by increasing IGF-1 gene expression.

Conclusions

The results of this study indicate that adding mulberry leaf powder to poultry feed at a concentration of 3 g/kg had the best effect on the expression activity of the growth hormone and insulin-like factor genes in broiler chickens. Therefore, we recommend using a higher concentration as a feed additive**.** For a better understanding, other hormones such as MyoD can be studied or histological sections of the intestine can be made and the type of microorganisms in the intestine after addition.

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Conflict of interest

The authors reveal that there is no conflict of activity

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