Journal of Applied Veterinary Sciences, 9 (1): 105-114 (January 2024).
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

The role of *Azolla pinnata* in hepatic protection and immunity stimulation in broiler chickens

Mohamed S. Hamouda[1], Hoda A. Mahrous[2], Hanafy A. Hamza[2], Gihan M. El Moghazy[1*], and Mohammed H. Abdel A'al[1]

[1]Regional Center for Food and Feed, Agricultural Research Center, Giza, Egypt
[2]Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Egypt
***Corresponding Author:** Gihan M. El Moghazy, **E-Mail:** g_rcff@hotmail.com

ABSTRACT

The aim of the present study was to estimate the phenolic compound and flavonoid contents in *Azolla pinnata (A. pinnata)* fern extract and evaluate its effect on the liver and immune system in broiler chicks. Mass spectrometry was used to identify the predominant active ingredients. The same study estimated the effect of the detected phytochemicals on the expression of some hepatic protection and immune stimulation-related genes. The experiment was performed using 150 one-day-old Indian River chicks, which were divided into five groups, each containing three replicates. The groups under study were as follows: T1 was fed a corn-based diet with no supplements, T2 was fed a corn-based diet supplemented with 5% *A. pinnata* sundried fern, T3 was fed a corn-based diet supplemented with 10% *A. pinnata* sundried fern, T4 was fed a corn-based diet supplemented with 15% *A. pinnata* sundried fern, and T5 was fed a corn-based diet supplemented with 20% *A. pinnata* sundried fern. At the end of the experiment, three birds from each subgroup were slaughtered, and their livers were collected to estimate the expression of SOD1, CAT, ACC, LPL, IL8, IL10, and TLR2 genes using real-time PCR. The obtained results showed that all treatments had a significant effect on the tested genes, as they caused up-regulation of their expression, indicating that these genes have antioxidant and immunostimulatory effects. More research is needed to correlate the recommended inclusion rates of *A. pinnata* with other performance parameters to achieve the best economic production strategies

Original Article:
DOI:https://dx.doi.org/10.21608/javs023.249784.1293
Received : 19 November, 2023.
Accepted :22 December, 2023.
Published in January, 2024.

Keywords: *Azolla pinnata*, phenolic compounds, flavonoid, hepatic protection, PCR and gene expression.

J. Appl. Vet. Sci., 9(1): 105-114.

INTRODUCTION

Azolla is a genus of aquatic ferns belonging to the family *Salviniaceae* (**Rashad, 2021)**. These ferns are highly specialized and have a unique appearance, resembling duckweed or mosses rather than typical ferns. One of its remarkable features is its fast growth, attributed to a symbiotic relationship with a cyanobacterium that enables Azolla to fix nitrogen from the atmosphere **(Nayak and Padhy 2017)**. This versatile aquatic plant has applications ranging from its use as a feed supplement for livestock to its potential role in sustainable agriculture and water purification (**Sońta *et al*., 2019)**. *Azolla pinnata*, a species of aquatic fern, has been subjected to compositional analysis to understand its nutritional content. Chemical analyses have revealed that *A. pinnata* contains significant amounts of nutrients. Azolla species can be used as a source of protein and other essential nutrient elements for poultry species **(Abd El-Ghany, 2020).** Azolla is rich in crude protein, ranging from approximately 12.6% to 20.20% of dry matter (**Swain *et al*., 2022)**. Other components such as amino acids like glutamic acid, aspartic acid, leucine, alanine, and arginine have also been identified in Azolla. Additionally, its composition includes crude fibre, ether extract, ash, and carbohydrates making it a potential source of nutrients for various applications, including livestock feed (**Refaey *et al*., 2023)**. The inclusion of Azolla at 15, 30 and 45% in broiler feed has a good effect on body weight and FCR, with no adverse effect on the normal physiology of broilers. Also, supplementation with Azolla can decrease production costs in the broiler industry by more than 30% (**AL-Shwilly**, **2022)**. *A. pinnata* has been found to possess significant antioxidant activity, which can be attributed to its high content of

phenols and flavonoids (**Kamel and Hamed, 2021)**. Studies indicate that *A. pinnata* exhibits potent antioxidant efficacy due to the presence of these phytochemicals. Phenolic compounds and flavonoids are known for their ability to scavenge free radicals and reduce oxidative stress (**Thiripurasundari and Padmini 2018).**

The higher levels of phenolic and flavonoid content in *A. pinnata* contribute to its potential protective and nutritional value. The antioxidant properties of *A. pinnata* make it a valuable natural source for combating oxidative damage and promoting overall health **(Nawaz *et al*., 2014)**. Also, *A. pinnata* has shown potential as an immunostimulant, exhibiting effects that can modulate the immune response in various organisms. Studies suggest that *A. pinnata* may have immune-modulatory effects, enhancing cell-mediated immune responses (**Mishra *et al*., 2016)**. It has been noted that feeding different levels of *A. pinnata* to turkey can lead to improved immune-related parameters (**Shukla *et al.,* 2018**) due to the presence of bioactive compounds such as antioxidants, anti-inflammatory agents, and other phytochemicals in *A. pinnata* that have a direct effect as a potential immunostimulant (**Abd Elrasoul *et al.,* 2020**). This aquatic fern's role as an immunostimulant highlights its significance not only in sustainable agriculture and aquaculture but also in potential nutritional applications aimed at boosting immune functions and overall health **(Lumsangkul *et al.,* 2022)**. The aim of this study is to evaluate the role of *A. pinnata* in broiler nutrition and estimate its effect on hepatoprotection and immune stimulation-related genes.

MATERIALS AND METHODS

Birds and experimental design

The experiment was carried out at Faculty of Agriculture, Benha University, Egypt, following the ethics of using animals in laboratory work according to EU (2010) **and SAIRS (2023**. A total of 150 one-day-old Indian River chicks were divided into 5 treatments, each consisting of 3 replicates. Each replicate contained 10 birds, so all treatments had a similar weight at the beginning of the experiment (43±1g). The chicks in all treatments were kept under similar hygienic and environmental conditions and vaccinated against: a) infectious bronchitis (IB) by coarse spray and Newcastle (NC) and Gumboro (G) by subcutaneous injection on the 1st day; b) NC and avian influenza (AI) by subcutaneous injection on the 5th day; c) NC and IB by eye drops on the 7th day; d) G in drinking water on the 14th day; and finally e) NC in drinking water on the 14th and 25th days. The chicks were housed on the floor with wire borders under continuous fluorescent lighting (10 watts/m2) and provided with an unmedicated corn-soybean-based meal diet (containing no added antibiotics, Coccidiostat, or growth promoters) and water *ad libitum*.

Treatments

The diet was prepared based on the Indian River Broiler Management Handbook **(Aviagen, 2018),** as illustrated in Tables 1 and 2, including only beginner and grower diets as recommended by the handbook. The untreated diet was fed to the T1 group (the control group), while T2, T3, T4 and T5 were fed a supplemented diet in which *A. pinnata* replaced soybean meal in complete formulated feed in percentages of 5%, 10%, 15% and 20%, respectively.

At the end of the experiment (35 days), 3 individuals from every replicate were sacrificed, and liver samples were collected under complete aseptic conditions and kept on ice until reaching the laboratory, where the samples were stored at -80 C^{o} for further analysis.

Table 1: Diet ingredients and calculated chemicals composition of starter diet.

Feed stuff	Starter				
	T1	T2	T3	T4	T5
Maize %	46.5	44.3	46.8	47	46.8
Soybean meal 44%	21.8	20.7	19.6	18.5	17.4
Corn bran %	10	10.1	5	9.9	12.6
Azolla %	0	2.2	4.4	6.6	8.8
Bone meal %	0	1	0	0.8	0
Sodium chloride %	1	1	1	1	1
Soy oil %	5	5	5	5	5
*Premix %	1	1	1	1	1
Concentrate (52%)	14.5	14.5	12	10	8
Methionine %	0.1	0.1	0.1	0.1	0.1
Lysine %	0.1	0.1	0.1	0.1	0.1
Total	100	100	100	100	100
Chemical analysis					
Crude protein (%)	22.77	23.10	23.22	23.00	23.07
Metabolizable Energy (kcal/kg)	3200	3200	3200	3200	3200

Where: T1: control, T2: 5%Azolla, T3: 10% Azolla, T4: 15% Azolla, T5:20%Azolla. *Each3.0 Kg of the of vitamins, trace minerals premix contains vitamin A 12000000 IU, vitamin D3 2200000 IU, vitamin E 10000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1600 mg, vitamin B12 10 mg, Niacin-30000 mg, Calcium-D-Pantothenic acid 10000 mg, Biotin-50 mg, Folic Acid-1000 mg and Choline 250000 mg, vitamin K-4000mg, antioxidant,10g, Trace mineral mixture: Iron 30000 mg, Iodine 1000 mg, Copper 10000 mg, Manganese 60000 mg, Zn 50000 mg, Selenium 100 mg, Cobalt 100 mg, and carrier (Calcium Carbonate) up to 3kg. Concentrated mixture of vitamins, trace (micro) minerals or diluents which may contain other feed additives such as amino acids.

Table 2: Diet ingredients and calculated chemicals composition of grower diet.

Feed stuff	Grower				
	T1	T2	T3	T4	T5
Maize %	47.3	43.3	51	43.3	42
Soybean meal 44%	19.5	18.5	17.5	16.5	15.5
Corn bran %	15	17	9.8	17	17
Azolla %	0	2	4	6	8
Bone meal %	1	5	1.5	3	4.3
Salt %	1	1	1	1	1
Oil %	5	5	5	5	5
*Premix %	1	1	1	1	1
Concentrated (52)	10	7	9	7	6
Methionine %	0.1	0.1	0.1	0.1	0.1
Lysine %	0.1	0.1	0.1	0.1	0.1
Total	100	100	100	100	100
Chemical analysis					
Crude protein (%)	20.30	20.15	20.11	20.02	19.94
Metabolizable Energy (kcal/kg)	3287	3240	3200	3225	3200

Where: T1: control, T2: 5%Azolla, T3: 10% Azolla, T4: 15% Azolla, T5:20%Azolla. *Each3.0 Kg of the of vitamins, trace minerals premix contains vitamin A 12000000 IU, vitamin D3 2200000 IU, vitamin E 10000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1600 mg, vitamin B12 10 mg, Niacin-30000 mg, Calcium-D-Pantothenic acid 10000 mg, Biotin-50 mg, Folic Acid-1000 mg and Choline 250000 mg, vitamin K-4000mg, antioxidant,10g, Trace mineral mixture: Iron 30000 mg, Iodine 1000 mg, Copper 10000 mg, Manganese 60000 mg, Zn 50000 mg, Selenium 100 mg, Cobalt 100 mg, and carrier (Calcium Carbonate) up to 3kg. Concentrated mixture of vitamins, trace (micro) minerals or diluents which may contain other feed additives such as amino acids.

Estimation of gene expression in the liver

Estimation of the effect of the additive on some hepatoprotection responsible genes was performed by measuring the expression of Superoxide dismutase 1 (SOD1), Catalse (CAT), Acetyl-CoA carboxylase alpha (ACC) and Lipoprotein lipase (LPL) genes. Also, the expression of Interleukin-8 (IL8), Interleukin-10 (IL 10) and Toll-like receptor 2 (TRL2) was determined as an indicator about its effect on immunity parameters. Molecular studies were performed as follow:

RNA Extraction

Total RNA was extracted from liver tissue using Trizol Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) according to the manufacturer's protocol.

RNA assessment

Using a (Nano Drop 1000, USA) spectrophotometer, RNA content and purity in extracted samples were evaluated. The concentration of nucleic acids was precisely measured using the absorbance at 260 nm, as well as at 280 nm.

Reverse transcription

Reverse transcription, which uses the extracted RNA as a template to create cDNA, comes after RNA extraction and quality checks. Utilizing the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific / Ferments) in accordance with the manufacturer's instructions, the reverse transcriptase enzyme uses the RNA template and short-sequence primers to direct the synthesis of the first strand cDNA, which is then utilized as a template for the qPCR reaction. The obtained cDNA was kept at -80^{o}C.

Quantitative Real-Time PCR

A total of four biological replicates of each RT-qPCR reaction were performed. Superoxide dismutase 1 (SOD 1), Catalase (CAT), Acetyl CoA carboxylase (ACC), Lipoprotein lipase (LPL), Interleukin 8 (IL8), Interleukin 10 (IL10), toll-like receptor 2 (TLR2), and beta-actin reference genes were included in the cytokine gene panel. These genes were used to normalize the data. Table 3 lists the oligonucleotide sequences of the primers according to **Ahmadipour *et al., (*2018)**. RT-qPCR reactions were conducted with a total volume of 20 µL. The reaction mixture included 12.5 µL of Maxima SYBR Green qPCR Master Mix (Thermo Scientific/Fermentas, Vilnius, Lithuania), one µM of each primer, and 2 µL of diluted cDNA (70 ng/µl). Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The thermal program included a step of initial denaturation (15 min at 95 ^{o}C), followed by 40 cycles of denaturation (10 s at 95 ^{o}C), annealing (15 s at 58 ^{o}C), and extension (30 s at 72 ^{o}C). Fluorescence was measured at the end of each extension step. After completing the thermal program, the melting curve was generated, which indicated amplification specificity. The thermal program for the melting curve included a gradual increase in the temperature up to 98^{o}C and measuring the fluorescence of the melting amplicon.

Statistical analysis

Analysis of data was carried out using GLM with SAS software (version 9.4, SAS Institute Inc., Cary, NC, 2014). Results with a P value $\leq$ 0.05 were considered as significantly different. Mean separations were carried out using Duncan. All data were expressed as the mean ± SE for each treatment. The relative transcript levels and the fold changes in transcript abundance were calculated using efficiency adjusted Pfaffl methodology **(Pfaffl, 2001)**.

Table 3: primer sequences used for quantitative real time PCR analysis of broiler liver.

Target	Forward primer	Reverse primer	Accession No.
ß-actin	AGCGAACGCCCCCAAAGTTCT	AGCTGGGCTGTTGCCTTCACA	NM_205518.1
SOD1	CACTGCATCATTGGCCGTACCA	GCTTGCACACGGAAGAGCAAGT	NM_205064.1
CAT	TGGCGGTAGGAGTCTGGTCT	GTCCCGTCCGTCAGCCATTT	NM_001031215.1
ACC	CAACGAGTCGGGCTACTACC	GGTCCTTGGTCACGTATGGG	J03541.1
LPL	CTCCGATCCCGAAGCTGAGATG	CTGTCCAGGAACCAGGTAGC	NM_205282.1
IL 8	GGCTTGCTAGGGGAAATGA	AGCTGACTCTGACTAGGAAACTGT	--
IL 10	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	--
TLR 2	TTAAAAGGGTGTGCCAGGAG	GTCCAAACCCATGAAAGAGC	AB050005

Lipoprotein lipase (LPL), Superoxide dismutase 1 (SOD 1), Catalase (CAT), acetyl CoA carboxylase (ACC), Interleukin 8(IL8), Interleukin 10 (IL10), Toll-Like receptor 2 (TLR2) and ß-actin (Beta Actin as Reference gene)

RESULTS

The intricate relationship between plant-derived flavonoids and phenolic compounds and their impact on liver protection and immunity in broilers has garnered significant attention. Flavonoids and phenolic compounds are integral components of various plants. Their potential as natural antioxidants and immune modulators has led to studies exploring their effects on broiler liver health and immune functions. These compounds have been reported to exhibit protective effects against hepatic injury potentially through their antioxidant properties. Furthermore, their immunostimulatory potential suggests their role in enhancing broiler immune responses. As interest in natural feed additives grows, understanding the effects of flavonoids and phenolic compounds on liver protection and immunity in broilers holds promise for improving poultry health and production.

From the data obtained in this research work and illustrated in Table 4, it was clear that *A. pinnata* extract contained many phenolic compounds and flavonoids with 9-Octadecenoic acid (Z), 3,4,2',4',6'-Pentamethoxychalcone, and 5-Hydroxy-7-methoxyflavone being the most predominant compounds as the result of Gas Chromatography Mass Spectrometry (GC-MS/MS) analysis.

Table 4: Peak area of detected active ingredients in *A. pinnata* sample under study

NO.	Retention Time	Name	Area Sum %
1	9.00	7,2',3'-Trimethoxyflavanone	2.89
2	10.26	2'-Hydroxy-2,3,4'-trimethoxychalcone	5.16
3	12.37	3,4,2',4',6'-Pentamethoxychalcone	20.86
4	13.45	5-Hydroxy-7-methoxyflavone	15.03
5	14.49	9-Octadecenoic acid (Z)	39.17
6	16.55	3,4-Dimethoxy-2'-(acetyl)oxy-5'-methylchalcone	9.40
7	18.78	Kaempferol 3-methyl ether	0.81
8	20.44	4',6'-Dimethoxy-2'-hydroxy-4-methylchalcone	0.40
9	21.52	3',4',5,7-Tetrahydroxy-3-methoxyflavone	6.28

From Tables 1 and 2, it is clear that feeding different formulae to the groups under study had a significant effect on the expression of the investigated hepatoprotective and immunity-related genes. It was clear that the higher the inclusion rate of *A. pinnata* in the diet, the greater the gene expression of some tested traits.

The increase in the inclusion rate of Azolla in the diet caused increased expression of SOD1, CAT, LPL, IL8, IL10, and TLR2 in all treatments when compared to the control group (T1), except for T2, which was fed Azolla at a concentration of 5% and showed lower values compared to T1 for all genes except ACC and IL10 (Table 5; Figures 1:3). Values of gene expression in T5 showed the highest response to the increased amount of Azolla, as recorded in the values of SOD1, IL8, and TLR2, whereas T3 showed the best results in the cases of CAT and LPL. T2 showed the best result for IL10 gene expression. As the lower expression of the ACC gene is

considered a positive result as it indicates lower lipogenesis and lower fat deposition, T2 showed the best response, which was indicated by the lowest ACC gene expression.

A gradual and significant increase in the expression of SOD1 and LPL started in T3, which then increased with an increase in the inclusion rate of Azolla. In contrast, a significant increase in the expression of CAT started at T3, followed by a gradual significant decrease with higher inclusion rates of the supplement. The expression of ACC started to decrease in T2 followed by a significant gradual increase in both T3 and T4, and T5 indicating that the active ingredient of the used supplement succeeded in downregulating the ACC gene only at a 5% inclusion rate.

Table 5: Effect of *A. pinnata* on gene expression in the liver of broiler chickens measured at 35 day of age (%).

Gene	Dietary levels of *A. pinnata* %				
	Control	5%	10%	15%	20%
SOD1	$0.99^{d} \pm 0.36$	$0.38^{e} \pm 0.01$	$2.02^{c} \pm 0.68$	$2.17^{b} \pm 0.61$	$5.83^{a} \pm 0.22$
CAT	$0.98^{d} \pm 0.17$	$0.72^{e} \pm 0.23$	$2.17^{a} \pm 0.12$	$2.06^{b} \pm 1.59$	$1.92^{c} \pm 0.10$
ACC	$1.15^{d} \pm 0.72$	$1.79^{c} \pm 0.24$	$2.83^{b} \pm 3.11$	$2.83^{b} \pm 1.88$	$4.33^{a} \pm 1.28$
LPL	$0.91^{d} \pm 0.33$	$0.48^{e} \pm 0.05$	$2.68^{a} \pm 0.26$	$2.01^{b} \pm 0.85$	$1.68^{c} \pm 0.20$
IL8	$0.98^{b} \pm 0.19$	$0.53^{e} \pm 0.19$	$0.78^{d} \pm 0.02$	$0.82^{c} \pm 0.46$	$1.67^{a} \pm 0.39$
IL10	$1.08^{c} \pm 0.08$	$1.21^{a} \pm 0.22$	$0.88^{e} \pm 0.37$	$0.93^{d} \pm 0.19$	$1.10^{b} \pm 0.60$
TLR2	$0.98^{b} \pm 0.04$	$0.88^{d} \pm 0.21$	$0.87^{d} \pm 0.15$	$1.02^{b} \pm 0.10$	$4.96^{a} \pm 2.28$

Superoxide dismutase 1 (SOD 1), Catalase (CAT), Acetyl CoA carboxylase (ACC), Lipoprotein lipase (LPL), Interleukin 8(IL8), Interleukin 10(IL10), Toll-Like receptor 2 (TLR2), $^{a, b, c}$ Means in the same raw with different superscripts are significantly different ($P < 0.05$) and standard error.

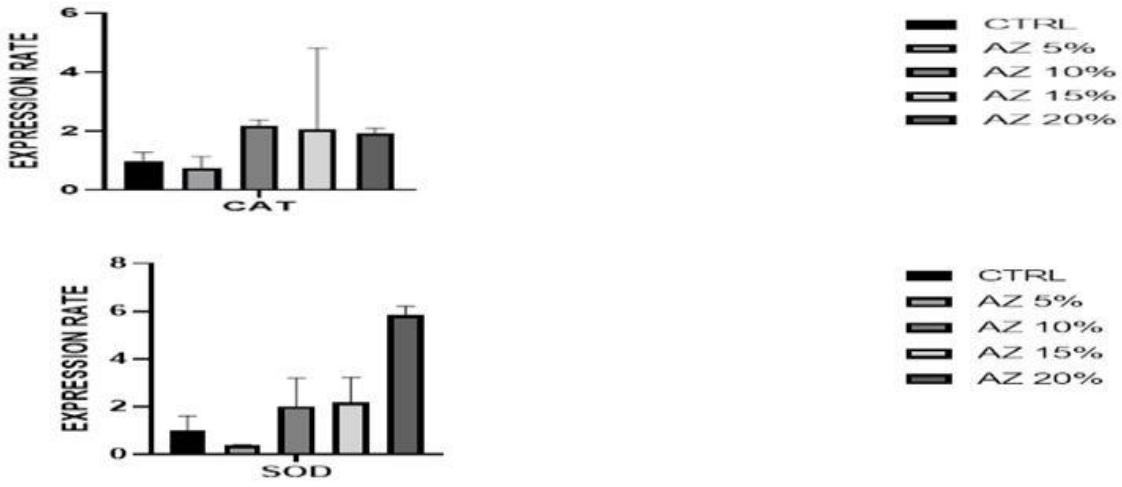

Fig 1: Effect of Azolla on genetic antioxidant response markers (CAT and SOD) in broilers chicken liver genes.

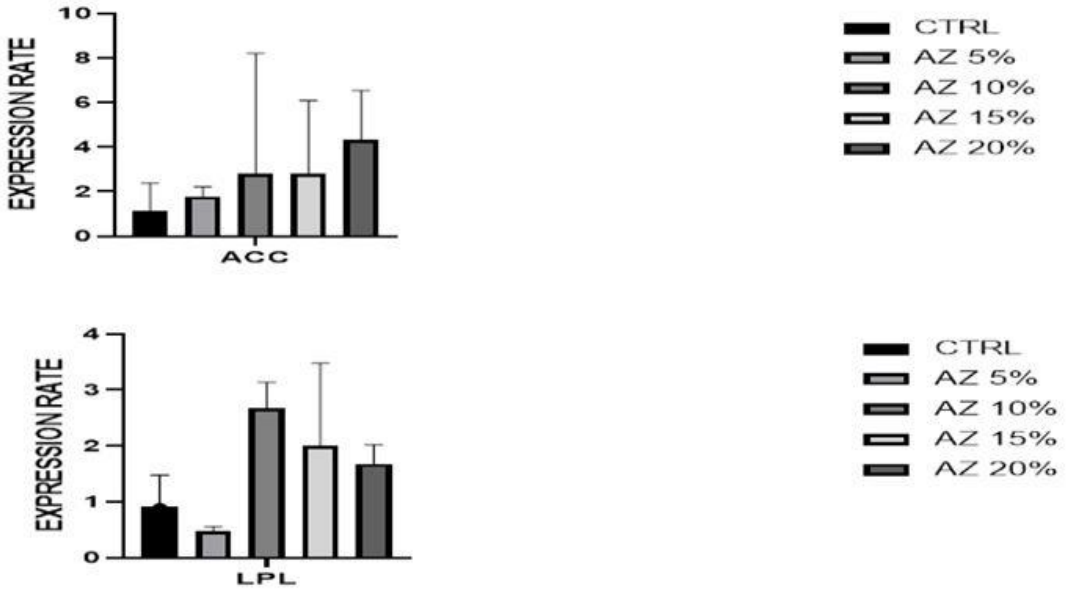

Fig 2: Effect of Azolla on genetic antioxidant response markers (ACC and LPL) in broilers chicken liver genes.

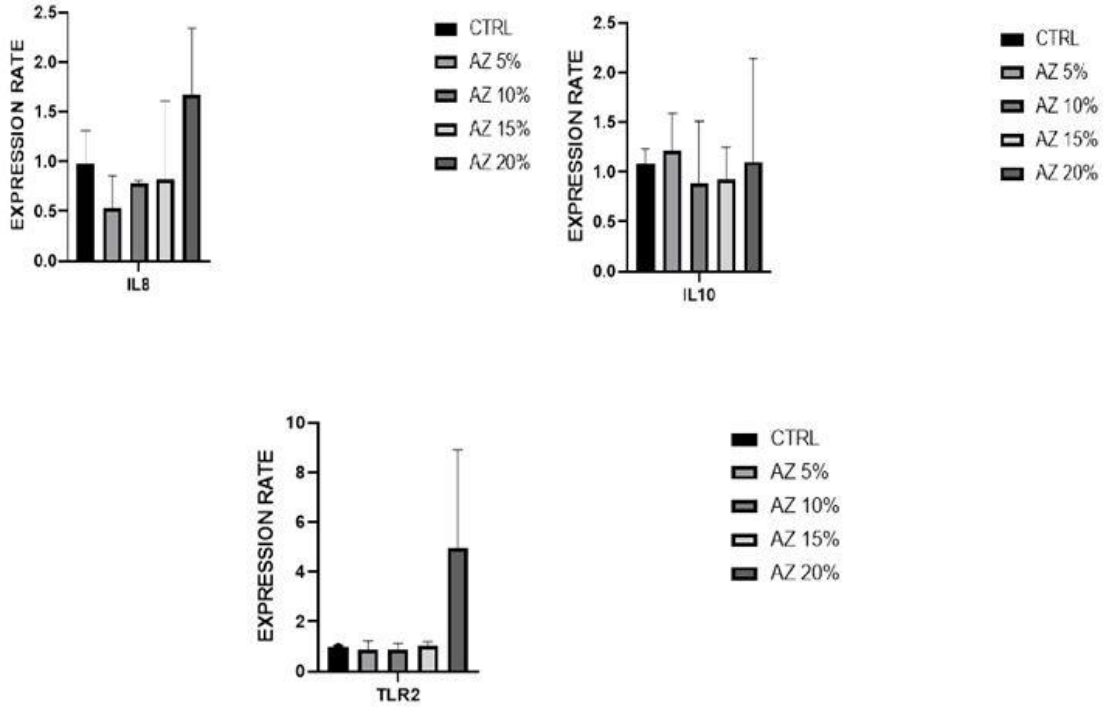

Fig 3: Effect of Azolla on genetic immune response markers (IL8, IL10 and TLR2) in broiler chicken liver genes.

DISCUSSION

This result agrees with that reported by **Sreenath *et al.*, (2016)** and **Nayak and Padhy (2017)** who detected 9-Octadecenoic acid (Z), 3,4,2',4',6'-Pentamethoxychalcone, and 5-Hydroxy-7-methoxyflavone as the predominant phytogenetic compounds in *A. pinnata* extract. The effect of *A. pinnata* in broiler nutrition on hepatoprotection and immune stimulation-related genes can be explained by these points. The LPL gene, encoding lipoprotein lipase (LPL), plays a fascinating role in poultry, acting as a regulator of fat metabolism by controlling the flow of fat into adipose tissue (storage) and muscle tissue (energy). It is found on the endothelial surface of capillaries and is responsible for breaking down circulating triglycerides in lipoproteins. LPL plays a major role in lipid transport, hydrolyzing

triglycerides into fatty acids for energy utilization and storage. It is highly expressed and active in tissues like adipose tissue and muscle **(Kumari *et al.*, 2021)**. *Azolla* flavonoids might impact lipoprotein lipase (LPL), as it is known to have antioxidant and cholesterol-lowering properties, thereby influencing triglyceride metabolism and cholesterol levels (**Pirahanchi *et al*., 2023**) and that was clear at inclusion rates of 10, 15 and 20%.

Superoxide dismutase 1 (SOD1) plays a crucial role in poultry as it is a key player in the antioxidant defense system, protecting cells from damage caused by reactive oxygen species (ROS) such as superoxide radicals. SOD1 has been extensively studied due to its significance in oxidative stress management and its involvement in various disease processes. It functions as a homodimer that binds copper and zinc, facilitating its antioxidant activity (**Wang *et al.*, 2018**). *Azolla* extracts have been studied for their antioxidant effects. Flavonoids, as potent antioxidants, are likely to influence the activity of SOD1 in the defense against oxidative stress by reducing reactive oxygen species (ROS) levels. It is plausible that the antioxidant properties of *Azolla* flavonoids could impact the activity of SOD1, helping to counteract oxidative damage in cells and tissues (**Egea *et al.*, 2020)**. The best result of SOD1 expression was obtained at a 20% inclusion rate followed by 15%, and then 10%.

Catalase gene expression in poultry has been a subject of research, particularly in broiler chickens. Studies have investigated the effects of various factors such as dietary supplementation and thermal manipulation on catalase expression. Research suggests that catalase (CAT) is an important antioxidant enzyme that helps mitigate oxidative stress in birds. Dietary supplementation with catalase has been explored to alleviate oxidative stress induced by toxins like deoxynivalenol. Flavonoids may influence the expression and activity of antioxidant enzymes like Catalase, contributing to enhanced antioxidant defence mechanisms in poultry (**Abd Elrasoul *et al.*, 2020**). Also, it was found that flavonoid-rich extracts from *A. pinnata* have shown antioxidant, anti-inflammatory, and anti-apoptotic effects (**Jafri *et al.*, 2022**). In this research work, it was clear that this protection, manifested by an increase in the expression of this gene, was gradually increasing, starting at 10%, followed by 15%, and then a 20% inclusion rate.

ACACA encodes the **Acetyl-CoA carboxylase alpha (ACCα) gene,** which plays a pivotal role in broiler growth and fat deposition, making it a crucial factor in broiler production and considered a key enzyme in fatty acid synthesis. Researchers had investigated the effects of silencing ACACA, its expression patterns in different tissues and ages, and its role in de novo lipid biosynthesis. The gene's expression is tissue-specific, being found in heart, muscle, liver, and colon tissues. Understanding ACACA gene expression provides insights into lipid metabolism regulation and the potential for manipulating lipid-related traits in poultry through gene silencing or modulation. Phenolic compounds and flavonoids found in plants have been extensively studied for their potential bioactive properties, including anti-inflammatory effects (**Liu *et al.*, 2023)**. It's plausible that these compounds could potentially influence gene expression as part of their regulatory activities (**Mahfouz *et al.*, 2021)**. But in this study, there was no enhancing effect by the addition of *A. pinnata* with all tested inclusion rates which was clear through the increase of ACACA gene expression by the increase of the inclusion rate, the reason for which can be attributed to the difference in the plant variety and its composition of active ingredients.

Interleukin 8 (IL-8) gene expression in poultry, specifically broiler chickens, has been studied in the context of infection and immunity. IL-8 is a pro-inflammatory chemokine that plays a role in triggering local inflammatory reactions and attracting immune cells to sites of infection. Researchers have analyzed the correlation between IL-8 and other interleukins, such as interleukin-6, in various tissues including the spleen and cecal tissues (**Elnagar *et al.*, 2021).** Phenolic compounds and flavonoids found in plants, including *Azolla*, have been studied for their potential anti-inflammatory effects and modulation of immune system function. It's known that certain phenolic compounds and flavonoids can influence the expression of pro-inflammatory cytokines, such as IL-8. These bioactive compounds often exhibit antioxidant and anti-inflammatory properties that may contribute to the regulation of IL-8 and other immune-related factors (**Chagas *et al.*, 2022)**. The best result was obtained in feed that contained 20% of *A. pinnata* in this study.

Interleukin 10 (IL-10) gene expression in poultry, particularly chickens, has been studied to understand its function in immune responses. Chicken IL-10 mRNA expression has been detected in various tissues, like the thymus, liver, and lung. This cytokine is involved in regulating immune responses and inflammatory processes. IL-10 production has been observed in chicken intestinal epithelial cells stimulated by specific antigens. Phenolic compounds and flavonoids found in plants, including *Azolla*, have been studied for their potential

anti-inflammatory effects and modulation of immune system function. It's known that certain phenolic compounds and flavonoids can influence the expression of cytokines, such as IL-10, that are involved in immune response regulation (**Kamboh *et al.*, 2016**). These bioactive compounds often exhibit antioxidant and anti-inflammatory properties that may contribute to the modulation of IL-10 and other immune-related factors which was clear in groups fed a diet supplemented with 5% *A. pinnata* and gave the best expression response of this gene.

TLR2 is expressed in various cell types, and its expression has been studied in response to different infections. Studies indicate that phytogenic feed additives can influence TLR2 gene expression. Research on TLR gene families, including TLR2, has highlighted their role in avian immunity **(Rehman *et al.*, 2021)**. This receptor is involved in recognizing pathogen-associated molecular patterns, contributing to the host's immune defence mechanism against various pathogens. It is known that some phenolic compounds and flavonoids can modulate the activity of toll-like receptors, including TLR2, which are crucial components of the innate immune response. These compounds can influence immune signaling pathways, inflammation, and cytokine production. Their anti-inflammatory and antioxidant properties suggest that they may play a role in regulating TLR2-mediated immune responses **(Pérez-Cano *et al.*, 2014).**

The best output of these effects was obtained in groups fed an experimental diet containing *A. pinnata* at a concentration of 20%, followed by 10%. These results agree with **Prabina and Kumar (2010); Islam (2017); Kamel and Hamed (2021); and Ibrahim *et al.*, (2023)** who reported the positive effect of *A. pinnata* on hepatic protection and immunity status by up-regulating antioxidant-responsible genes and interleukins and down-regulating lipogenesis-responsible genes. The increase in the production of interleukins in groups fed *A. pinnata* at different inclusion rates may be attributed to the good quality, quantity and availability of its protein content.

CONCLUSION

From this work it can be concluded that, supplementation of feed with different concentrations of *A.* pinnata (5, 10, 15 and 20%) caused an improvement in the expression of hepatic protection-related gene(s) and immunostimulant-related gene(s) and can be relied on as a good source of phytochemicals. More research work is needed to correlate the recommended inclusion rates of *A. pinnata* with other performance parameters to reach the best economic production strategy.

Conflict of interests

The authors declare no potential conflict of interest.

REFERENCES

AL-SHWILLY, H. A. J. 2022. Azolla as a New Dietary Source in Broiler Feed: A Physiological and Production Study. Archives of Razi Institute, 77(6): 2175-2180.

ABD EL-GHANY, W. A. 2020. A review on the use of Azolla species in poultry production. J. World Poult. Res. 10(2): 378-384. https://dx.doi.org/10.36380/jwpr.2020.44.

ABD ELRASOUL, A. S., MOUSA, A. A., SAHAR, H. ORABI, MOHAMED, M. A., GAD-ALLAH, S. M., ALMEER, R., ABDEL-DAIM, M. M., SHADEN, A. K., EL-SEEDI, H. R., and ABD ELDAIM, M. A., 2020. Antioxidant, Anti-Inflammatory, and Anti-Apoptotic Effects of *Azolla pinnata* Ethanolic Extract against Lead-Induced Hepatotoxicity in Rats. Antioxidants, 9: 1014. https://doi.org/10.3390/antiox9101014.

AHMADIPOUR, B., HASSANPOUR, H., and KHAJALI, F., 2018. Evaluation of hepatic lipogenesis and antioxidant status of broiler chickens fed mountain celery. BMC veterinary research, 14(1):1-7. https://doi.org/10.1186/s12917-018-1561-6.

AVIAGEN, 2018. Indian River Broiler Management Handbook.

CHAGAS, M. D. S. S., BEHRENS, M. D., MORAGAS-TELLIS, C. J., PENEDO, G. X. M., SILVA, A. R., and GONÇALVES-DE-ALBUQUERQUE, C. F., 2022. Flavonols and Flavones as Potential Anti-Inflammatory, Antioxidant, and Antibacterial Compounds. Oxid Med Cell Longev. 2022 Sep 6; 2022:9966750. https://doi.org/10.1155/2022/9966750.

EGEA, G., JIMÉNEZ-ALTAYÓ, F., and CAMPUZANO, V., 2020. Reactive Oxygen Species and Oxidative Stress in the Pathogenesis and Progression of Genetic Diseases of the Connective Tissue. Antioxidants 9, 1013. https://doi.org/10.3390/antiox9101013.

ELNAGAR, R., ELKENANY, R., and YOUNIS, G., 2021. Interleukin gene expression in broiler chickens infected by different *Escherichia coli* serotypes. Vet World. Oct;14(10):2727-2734. https://doi.org/10.14202%2Fvetworld.2021.2727-2734.

EUROPEAN UNION DIRECTIVE., 2010. The protection of animals used for scientific purposes: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063

IBRAHIM, S., ATEYA, A., and ABDO, M., 2023. Economic Evaluation of Using Azolla on Growth Performance of Broiler Chickens: Gene Expression Impact. Egypt. J. Vet. Sci. 55(1): 29-40. https://doi: 10.21608/EJVS.2023.215995.1519

ISLAM, M. A., (2017). Effect of Azolla (*Azolla Pinnata*) on Growth and Lipid Profiles of Broiler Chickens. Annals of Bangladesh Agriculture 21 (1 & 2): 73–78.

JAFRI, S. A. A., KHALID, Z. M., KHAN, M. Z., and JOGEZAI, N., 2022. Evaluation of phytochemical and antioxidant potential of various extracts from traditionally used medicinal plants of Pakistan. Open Chemistry, vol. 20, no. 1, 2022, pp. 1337-1356. https://doi.org/10.1515/chem-2022-0242.

KAMBOH, A. A., HANG, S. Q., KHAN, M. A., and ZHU, W. Y., 2016. *In vivo* immunomodulatory effects of plant flavonoids in lipopolysaccharide-challenged broilers. Animal. 2016 Oct;10(10):1619-25. https://doi.org/10.1017/S1751731116000562.

KAMEL, E. R., and HAMED, E., 2021. Effect of dried Azolla on growth performance, hematological, biochemical, antioxidant parameters, and economic efficiency of broiler chickens. Adv. Anim. Vet. Sci., 9(11): 1886-1894. http://dx.doi.org/10.17582/journal.aavs/2021/9.11.1886.1894.

KUMARI, A., KRISTENSEN, K. K., PLOUG, M., and WINTHER, A. L., 2021. The Importance of Lipoprotein Lipase Regulation in Atherosclerosis. Biomedicines. Jul 6;9(7):782 https://doi.org/10.3390/biomedicines9070782.

LIU, S., LAI, J., FENG, Y., ZHUO, Y., ZHANG, H., CHEN, Y., LI, J., MEI, X., ZENG, Y., SU, J., DENG, Y., JIANG, F., YANG, S., TAN, H., HON, C. T., WEI, S., HAN, Z., WANG, F., and ZHONG, W., 2023: Acetyl-CoA carboxylase 1 depletion suppresses de novo fatty acid synthesis and mitochondrial β-oxidation in castration-resistant prostate cancer cells. J Biol Chem. Jan;299 (1):102720. https://doi.org/10.1016/j.jbc.2022.102720.

LUMSANGKUL, C., LINH N. V., CHAIWAN, F., ABDEL-TAWWAB, M., DAWOOD, M., FAGGIO, C., JATURASITHA, S., and VAN DOAN, H., 2022. Dietary treatment of Nile tilapia (*Oreochromis niloticus*) with aquatic fern (*Azolla caroliniana*) improves growth performance, immunological response, and disease resistance against *Streptococcus agalactiae* cultured in bio-floc system Aquaculture Reports 24, 101114. https://doi.org/10.1016/j.aqrep.2022.101114.

MAHFOUZ, S., SHANG, Q., and PIAO, X., 2021. Phenolic compounds as natural feed additives in poultry and swine diets: a review. J Animal Sci Biotechnol 12, 48. https://doi.org/10.1186/s40104-021-00565-3.

MISHRA, D. B., ROY, D., KUMAR, V., BHATTACHARYYA, A., KUMAR, M., KUSHWAHA, R., and VASWANI, S., 2016. Effect of feeding different levels of *Azolla pinnata* on blood biochemicals, hematology and immunocompetence traits of Chabro chicken. Veterinary World, 9(2), 192. https://doi.org/10.14202/vetworld.2015.192-198.

NAWAZ, N. A. S., SYED, J., DILEEP, N., RAKESH, K. N., and PRASHITH KEKUDA, T. R., 2014. Antioxidant activity of *Azolla pinnata* and Azolla rubra–A comparative study. Sch Acad J Biosci, 2(10), 719-23.

NAYAK, N., and PADHY, R. N., 2017. GC-MS analysis of bioactive compounds and host-toxicity studies of *Azolla caroliniana* symbiotic with the cyanobacterium Anabaena azollae. Indian J Pharm Educ, 51(2S), S24-S33. https://doi.org/10.5530/ijper.51.2s.46.

PÉREZ-CANO, F. J., MASSOT-CLADERA, M., RODRÍGUEZ-LAGUNAS, M. J. and CASTELL, M., 2014. Flavonoids Affect Host-Microbiota Crosstalk through TLR Modulation. Antioxidants. 3, 649-670. https://doi.org/10.3390/antiox3040649.

PFAFFL M.W. 2001. Development and validation of an externally standardised quantitative insulin like growth factor-1 (IGF-1) RT–PCR using LightCycler SYBR® Green I technology. In Meuer,S., Wittwer,C. and Nakagawara,K. (eds), Rapid Cycle Real-time PCR, Methods and Applications. Springer Press, Heidelberg, Germany pp. 281–291. http://dx.doi.org/10.1007/978-3-642-59524-0_30.

PIRAHANCHI, Y., ANORUO, M. D., and SHARMA S., 2023. Biochemistry, Lipoprotein Lipase. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK537040/

PRABINA BJ., and KUMAR K., 2010. Dried Azolla as a nutritionally rich cost effective and immune - modulatory feed supplement for broilers. Asian J. Anim. Sci. 5: 20-22.

RASHAD, S., 2021. An overview on the aquatic fern Azolla spp. as a sustainable source of nutrients and bioactive compounds with resourceful applications. Egyptian Journal of Aquatic Biology and Fisheries, 25(1): 775-782. http://dx.doi.org/10.21608/ejabf.2021.150205.

REFAEY, M. M., MEHRIM, A. I., ZENHOM, O. A., AREDA, H. A., RAGAZA, J. A., and HASSAAN, M. S., 2023. Fresh Azolla, *Azolla pinnata* as a Complementary Feed for *Oreochromis niloticus*: Growth, Digestive Enzymes, Intestinal Morphology, Physiological Responses, and Flesh Quality. Aquaculture Nutrition, 2023. https://doi.org/10.1155/2023/1403704.

REHMAN, M. S., REHMAN, S. U., YOUSAF, W., HASSAN, F. U., AHMAD, W., LIU, Q., and PAN, H., 2021. The Potential of Toll-Like Receptors to Modulate Avian Immune System: Exploring the Effects of Genetic Variants and Phytonutrients. Front Genet. Aug 26; 12:671235. https://doi.org/10.3389/fgene.2021.671235

SHUKLA, M., BHATTACHARYYA, A., SHUKLA, P., ROY, D., YADAV, B., and SIROHI, R., 2018. Effect of Azolla feeding on the growth, feed conversion ratio, blood biochemical attributes and immune competence traits of growing turkeys. Veterinary World, 11 (8): 2231-0916. https://doi.org/10.14202/vetworld.2018.459-463.

SOŃTA, M., REKIEL, A., and BATORSKA, M., 2019. Use of duckweed (Lemna L.) in sustainable livestock production and aquaculture–a review. Annals of Animal Science, 19(2), 257-271. https://doi.org/10.2478/aoas-2018-0048.

SOCIETY FOR ANIMAL RIGHTS IN SCIENCE (SAIRS)., 2023. The Animals in Science Regulation Unit., the Animals (Scientific Procedures) Act 1986 in England, Scotland and Wales. https://www.gov.uk/government/collections/animals-in-science-regulation-unit

SREENATH, K. B., SUNDARAM, S., GOPALAKRISHNAN, V. K., and POORNIMA, K., 2016. Quantitative phytochemical analysis, *in vitro* antioxidant potential and gas chromatography-mass spectrometry studies in ethanolic extract of *Azolla microphylla*. Asian J. Pharm Clin. Res., 9(2): 318-323.

SWAIN, B. K., NAIK, P. K., and BEURA, C. K., 2022. Nutritive value of azolla as poultry feed-a review. Indian Journal of Animal Nutrition, 39(1): 1-11. http://dx.doi.org/10.5958/2231-6744.2022.00001.9.

THIRIPURASUNDARI, B., and PADMINI, E., 2018. Preliminary phytochemical screening and evaluation of antimicrobial and antioxidant activity of *Azolla pinnata*. Int J Recent Sci Res, 9(5F): 26924-26930. http://dx.doi.org/10.24327/ijrsr.2018.0905.2151.

WANG, Y., BRANICKY, R., NOË, A., and HEKIMI, S., 2018. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J Cell Biol. 2018 Jun 4;217(6):1915-1928. https://doi.org/10.1083/jcb.201708007.

How to cite this article:
Mohamed S. Hamouda, Hoda A. Mahrous, Hanafy A. Hamza, Gihan M. El Moghazy, and Mohammed H. Abdel A'al, 2024. The role of *Azolla pinnata* in hepatic protection and immunity stimulation in broiler chickens. Journal of Applied Veterinary Sciences, 9 (1): 105-114.
DOI: **https://dx.doi.org/10.21608/javs.2023.249784.1293**