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EFFECTS OF *CHLORELLA VULGARIS* SUPPLEMENTATION ON OXIDATIVE STRESS, ERYTHROGRAM, AND LEUKOGRAM PROFILES IN *GALLUS GALLUS DOMESTICUS* WITH MOLECULAR DOCKING ANALYSIS

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Abstract

This study evaluated the effects of *Chlorella vulgaris* supplementation on oxidative stress, erythrogram, and leukogram parameters in 378 *Gallus gallus domesticus* (hens) assigned to six groups: control (C) and experimental (G1-G5). Over 71 days, blood samples were analyzed for serum metabolites, plasma minerals, antioxidant markers [glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (TAC), protein carbonyl (PCO), malondialdehyde (MDA)], and hematological profiles. *Chlorella* supplementation significantly enhanced TAC, SOD, and GSH, while reducing MDA and PCO in a dose-dependent manner. Erythrocyte and leukocyte profiles also improved, indicating strengthened antioxidant defenses and immune responses, with higher doses exerting greater effects. Molecular docking further confirmed nutrient efficiency through interactions between isoquinoline and 3S3Q protein.

Introduction

It is projected that by 2050, there will be 9.8 billion people on the planet. In order to meet the need for food production and food security, pressure has been placed on the world's food systems. In addition, finding substitute protein sources for animal products in people's meals is a latent demand in order to accomplish the Sustainable Development Goals (SDGs) and support the population. Thus, there is a close relationship between microalgae and various SDGs, including their potential use as a source of dietary proteins (Olabi *et al.* 2023).

In addition to being crucial for the food, aquaculture, and animal nutrition sectors, microalgal biomass contains significant nutritional components with positive effects on human health (Abideen *et al.* 2025). Typically, microalgae consist primarily of protein (40-70%), with significant amounts of lipids (5-30%), carbohydrates (10-25%), minerals (5-25%), and pigments (1-5%) (Ambati *et al.* 2014). Moreover, they include saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFAs), such as the health-promoting docosahexaenoic acid (DHA) (Sohedein *et al.* 2020). Microalgae are regarded as future super food because of their superior nutritional profile, which includes a more complete amino acid profile and a variety of micronutrients when compared to traditional sources of typical plant proteins. This trend has gained traction in the market alongside certain natural and sustainable foods that are already partially used in people's diets (Vrenna *et al.* 2021).

Microscopic algae have many types and one of its important type is *Chlorella vulgaris* which is in ditches, ponds, and muddy puddles. *Chlorella vulgaris* is a single-celled green algae that

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grows easily and produces large amounts of protein, chlorophyll, lutein, and other important micronutrients (Jeon *et al.* 2012).

The goal of the current study was to assess how food supplementation with Chlorella vulgaris affected the erythrogram, leukogram, growth performance, and oxidative stress indicators in *Gallus gallus domesticus*. The specific goals were to: (i) ascertain how graded levels of *C. vulgaris* affected body weight and relative organ weights; (ii) evaluate its impact on haematological parameters, such as erythrocyte and leukocyte profiles; (iii) investigate its impact on antioxidant status using serum oxidative stress markers, such as MDA, PCO, TAC, SOD, CAT, and GSH; and (iv) investigate the potential interaction of a chosen bioactive compound with the target protein using molecular docking analysis.

Materials and Methods

Dried *Chlorella vulgaris* powder (Algomed *C. vulgaris* Microalgae Powder, supplied by Daraz.pk, Pakistan) was used directly in the experimental diets. Six treatments were randomly assigned to 378 laying hens (36 weeks old, 1295 ± 127 g). These hens were housed in 42 cages, each holding nine birds, with seven cages per treatment group. A 14-day adaptation period preceded the 9-week experimental phase conducted at the Institute of Zoology, BZ University, Pakistan (February-June). Ambient temperature and humidity were recorded thrice daily (0930, 1530, 1950 h), ranging between 26.25-35.78°C and 24.7-37.1%, respectively. The temperature humidity index (THI) was calculated using the formula:

$$THI=(1.8\times T+32)-[(0.55-0.0055\times RH)\times(1.8\times T-26)]THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]THI=(1.8\times T+32)-[(0.55-0.0055\times RH)\times(1.8\times T-26)]$$

where T is the mean ambient temperature in degrees Celsius and RH is the relative humidity in % (Ravagnolo *et al.* 2000). Birds were kept under a 16 L : 8 D light regime with ad libitum water (19 ± 4°C) and a daily feed allowance of 109 g/hen. Diets were formulated based (Torki *et al.* 2015) recommendations: 2800 Kcal/kg metabolizable energy and 14.95% crude protein. The corn-soybean basal diet was used to prepare six diets: control (C) and five levels of *C. vulgaris* (1-5 g/l).

At trial end, birds were fasted overnight; three hens per pen were weighed, blood-sampled via wing vein, and processed for hematology (heparinized) and serum (non-heparinized). Hens were then slaughtered, and kidneys, liver, and heart were excised, rinsed (0.9% NaCl), weighed, and stored at –80°C for CAT and MDA assays.

Prior to slaughter, the final body weight was noted. The difference between the starting and final weights was used to determine body weight gain. According to (Latif *et al.* 2021) the weights of the liver, kidney, and heart were measured, and the relative organ weights (ROW) were computed.

Using an automatic cell counter and hematocrit analyzer, whole blood was analyzed for RBCs, hemoglobin (Hb), hematocrit (PCV%), total and differential leukocyte and platelet counts (Buttarello 2004).

SOD activity was quantified using a pyrogallol auto-oxidation assay in the presence of 1 mM DEPTA and 50 mM Tris-cacodylate buffer at pH 8.5, as described by (Marklund and Marklund 1974) and modified by (Guvvala *et al.* 2017). Reaction mixtures included buffer, pyrogallol (20 mM in 10 mM HCl), and sample (blood or tissue). Absorbance was recorded at 420 nm using a UV/Vis spectrophotometer (Biochrom Alpha F36, Pakistan) over 180 seconds to calculate SOD activity via pyrogallol inhibition.

Isoquinoline alkaloid, a key secondary metabolite identified from *C. vulgaris*, was docked against Gallus metabolic protein 3S3Q, selected from a panel of digestible enzymes and metabolites. Binding affinities were evaluated after functional group optimization using BIOVIA Discovery Studio Visualizer (Khalid *et al.* 2025).

Results and Discussion

Compared to the control group (C), G4, G5, and G3 showed a significant ($p \leq 0.05$) increase in final body weight and weight gain, while G1 showed no significant difference (Table 1). *Chlorella vulgaris* showed a dose-dependent effect on organ weight, with significantly higher values at higher doses (G5), suggesting its potential as a growth-promoting agent (Table 2).

Table 1. Effects of graded levels of *Chlorella vulgaris* on initial body weight, final body weight, and body weight gain in laying hens.

Parameter group	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
C	133.80 ± 2.25a	143.10 ± 2.60c	8.65 ± 0.08c
G1	132.90 ± 2.65a	142.30 ± 2.60c	9.55 ± 0.08c
G2	132.80 ± 2.30a	144.20 ± 2.20c	11.30 ± 0.08c
G3	132.90 ± 2.60a	146.50± 1.70b	13.70 ± 0.08b
G4	133.70 ± 2.40a	151.50 ± 3.20a	17.65 ± 0.08a
G5	133.90 ± 3.20a	152.10 ± 2.95a	18.30 ± 0.08a

C: control group, G1: 1 g/l *C. vulgaris*, G2: 2 g/l *C. vulgaris*, G3: 3 g/l *C. vulgaris*, G4: 4 g/l *C. vulgaris*, G5: 5 g/l *C. vulgaris*. Data shown as means ± SEM. Within the same column, values with distinct superscripts indicate a significant difference ($p < 0.05$).

Table 2. Differences in the absolute and relative weight of several organs in groups of treated and control chickens.

Parameter group	Final body wt (g)	Absolute wt - Liver (g)	Absolute wt - Kidney (g)	Absolute wt - Heart (g)	Relative wt - Liver (%)	Relative wt - Kidney (%)	Relative wt - Heart (%)
Control	1700 ± 50	35.5 ± 1.3b	9.6 ± 0.4d	8.2 ± 0.3d	2.09 ± 0.08b	0.56 ± 0.03d	0.48 ± 0.02d
G1	1800 ± 55	42.5 ± 1.4a	12.1 ± 0.5a	10.3 ± 0.4a	2.36 ± 0.09a	0.67 ± 0.04a	0.57 ± 0.03a
G2	1850 ± 60	46.1 ± 1.2a	13.5 ± 0.6ab	11.5 ± 0.5ab	2.49 ± 0.10a	0.73 ± 0.04a	0.62 ± 0.03a
G3	1900 ± 65	50.3 ± 1.5a	15.2 ± 0.6a	13.1 ± 0.5a	2.65 ± 0.12a	0.80 ± 0.05a	0.69 ± 0.04a
G4	1855 ± 55	44.7 ± 1.2a	13.1 ± 0.6b	11.2 ± 0.4b	2.41 ± 0.09a	0.71 ± 0.04b	0.60 ± 0.03b
G5	2050 ± 80	55.5 ± 1.5a	17.2 ± 0.7a	15.4 ± 0.5a	2.71 ± 0.13a	0.84 ± 0.06a	0.75 ± 0.04a

C: control group, G1: 1 g/l C. *vulgaris*, G2: 2 g/l C. *vulgaris*, G3: 3 g/l C. *vulgaris*, G4: 4 g/l C. *vulgaris*, G5: 5 g/l C. *vulgaris*. The data is shown as means ± SEM. Within the same column, values with distinct superscripts indicate a significant difference ($p < 0.05$).

Significant improvements in hematological parameters were observed with increasing dosages of *C. vulgaris*, especially in G4 and G5. The greatest RBC count ($9.02 \pm 0.31 \times 10^6$/µl) was observed in G5 as compared to the control ($7.91 \pm 0.47 \times 10^6$/µl) (Table 3). TLC varied among groups, with the highest value observed in G5 ($9.00 \pm 0.32 \times 10^3$/µl) (Table 4).

Compared with Chlorella-treated groups, controls showed higher MDA (23.75 ± 1.65 nmol/ml) and PCO (7.70 ± 0.56 µmol/ml), with the greatest reductions in G5 and G1. TAC was significantly elevated in all treated groups, highest in G1 (112.1 ± 21.5 µmol/ml) and G5 (95.0 ± 5.80 µmol/ml; $p = 0.005$). CAT activity showed a non-significant increase ($p = 0.1$), while SOD and GSH rose markedly (Table 5, Fig. 1).

Table 3. Effects of *Chlorella vulgaris* supplementation on erythrogram parameters in laying hens.

Parameter group	RBCs (10^6/ul)	HB (g/dl)	PCV (%)	Platelets (10^3/ul)
Control	7.91 ± 0.47a	13.82 ± 0.50ab	41.78 ± 1.65ab	832.40 ± 34.80a
G1	5.12 ± 0.72c	10.95 ± 0.48c	33.10 ± 1.40c	426.80 ± 29.00c
G2	7.46 ± 0.42a	12.98 ± 0.30b	39.58 ± 0.81b	782.10 ± 55.00ab
G3	6.79 ± 0.22abc	12.19 ± 0.09bc	38.20 ± 0.22bc	778.60 ± 42.90ab
G4	8.12 ± 0.14a	14.10 ± 0.18a	41.35 ± 1.06b	808.30 ± 33.10ab
G5	9.02 ± 0.31a	15.34 ± 0.36a	45.16 ± 0.85a	852.00 ± 36.00a

C: control group, G1: 1 g/l C. *vulgaris*, G2: 2 g/l C. *vulgaris*, G3: 3 g/l C. *vulgaris*, G4: 4 g/l C. *vulgaris*, G5: 5 g/l C. *vulgaris*. RBCs: Red blood cells, HB: Hemoglobin, PCV: Packed cell volume. The data is shown as means ± SEM. Within the same column, values with distinct superscripts indicate a significant difference ($p < 0.05$).

Table 4. Effects of *Chlorella vulgaris* supplementation on leukogram parameters in laying hens.

Parameter group	TLC (103/ul)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)
Control	8.10 ± 0.55ab	22.50 ± 1.80b	65.50 ± 1.72c	5.90 ± 0.60a
G1	5.20 ± 1.05c	19.30 ± 0.95c	73.30 ± 1.12a	5.90 ± 0.60a
G2	8.40 ± 0.24ab	23.40 ± 1.85b	68.90 ± 1.70bc	5.10 ± 0.72a
G3	8.50 ± 0.72ab	23.50 ± 1.70b	69.50 ± 0.25bc	5.70 ± 0.90a
G4	8.30 ± 0.49ab	23.50 ± 1.60b	66.70 ± 1.58c	6.10 ± 0.72a
G5	9.00 ± 0.32a	26.50 ± 0.80a	66.50 ± 1.52c	5.90 ± 0.60a

C: control group, G1: 1 g/l C. *vulgaris*, G2: 2 g/l C. *vulgaris*, G3: 3 g/l C. *vulgaris*, G4: 4 g/l C. *vulgaris*, G5: 5 g/l C. *vulgaris*. TLCs: Total leukocyte counts. The data is shown as means ± SEM. Within the same column, values with distinct superscripts indicate a significant difference ($p < 0.05$).

Table 5. Effects of *Chlorella vulgaris* supplementation on serum oxidative stress and antioxidant markers in laying hens.

Parameters	C	G1	G2	G3	G4	G5	p-values
MDA (nmol/ml)	23.75 ± 1.65a	15.50 ± 2.20b	16.95 ± 0.95b	19.20 ± 0.93b	16.85 ± 1.35b	14.90 ± 0.90b	0.01
PCO (μmol/ml)	7.70 ± 0.56b	5.95 ± 0.24a	7.05 ± 0.48ab	9.50 ± 0.22c	6.30 ± 0.29a	6.15 ± 0.31a	0.001
TAC (μmol/ml)	37.5 ± 4.45a	112.1 ± 21.5c	62.50 ± 19.9ab	63.7 ± 20.10ab	80.6 ± 5.72bc	95.0 ± 5.80bc	0.005
SOD (U/ml)	158.0 ± 9.55ab	204.2 ± 23.10b	146.8 ± 14.6a	157.3 ± 13.20ab	196.6 ± 3.85b	210.5 ± 4.00b	0.04
CAT (U/ml)	3.85 ± 0.36	4.60 ± 0.55	5.10 ± 0.54	3.35 ± 0.59	3.40 ± 0.51	4.20 ± 0.52	0.1
GSH (μmol/ml)	7.20 ± 0.55a	9.10 ± 0.42ab	10.30 ± 0.24abc	10.95 ± 0.49bc	13.25 ± 2.28c	12.10 ± 2.00bc	0.02

C: control group, G1: 1g/l C. *vulgaris*, G2: 2 g/l C.*vulgaris*, G3: 3 g/l C.*vulgaris*, G4: 4 g/l C.*vulgaris*, G5: 5 g/l C. *vulgaris*. TLCs: Total leukocyte counts. The data is shown as means ± SEM. Within the same column, values with distinct superscripts indicate a significant difference ($p < 0.05$).

Isoquinoline showed the strongest docking affinity to cathepsin B-like carboxydipeptidase 3S3Q, an asymmetric monomer with 254 subunits, 323 amino acids, and 536 water molecules (Fig. 2). Associated residues include C1P [N~2~-(morpholin-4-ylcarbonyl)-N-[(3S)-1-phenyl-5-(phenylsulfonyl)pentan-3-yl]-L-leucinamide] represented as chain B, and acetate ions as chains C and D (Hökelek *et al*. 2010). Molecular docking was performed using AutoDock Vina, identifying glutamic acid 316, valine 317 and alanine 243 (Figs 3 and 4),) as binding sites. According to research, supplementing with *C. vulgaris* considerably raised both final body weight and weight gain when compared to the control. Its high levels of chlorophyll, vital amino acids,

calcium, phosphorus, iodine, manganese, iron, and vitamins A, B-complex, C67, and E63 are thought to be responsible for this impact (Safi *et al.* 2014) findings are supported (Xu *et al.* 2014). Higher dosages of *C. vulgaris* increased Hb, PCV, RBC, and platelet counts, according to the erythrogram study. This suggests that the chlorophyll and other bioactive components of the plant, such as vitamins and minerals, stimulate erythropoiesis. Similarly, (Kotrbáček *et al.* 2013), supplementing chickens with chlorella boosted their red blood cell counts dramatically because of its nutritional and antioxidant qualities. The chlorophyll, iron, vitamin B_{12}, and folate content of *Chlorella vulgaris* are well-documented stimulants of erythropoiesis, promoting RBC proliferation and maturation (Kotrbacek *et al*. 1994, Kotrbáček *et al.* 2013).

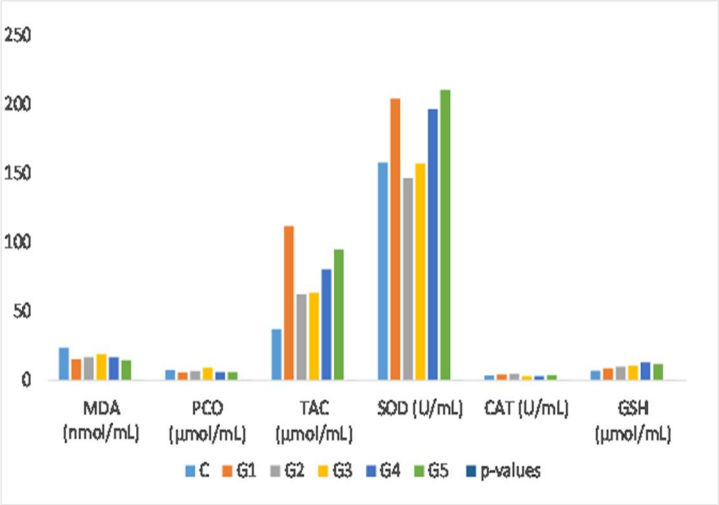

Fig. 1. Chickens' serum oxidative stress indicators Owing to C. *vulgaris* supplementation.

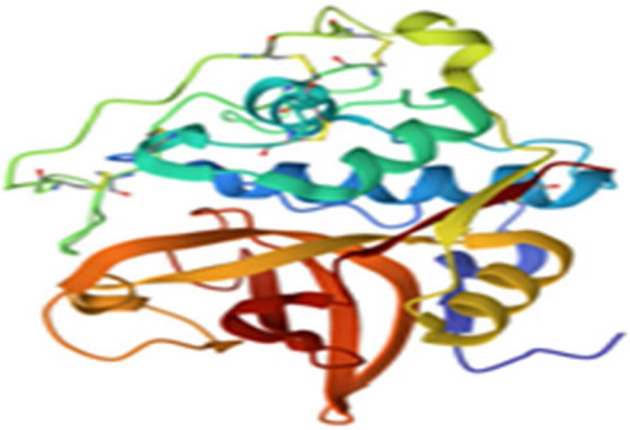

Fig. 2. 3S3Q protein structure, red bonds, amino acids residues, green bonds, carbon-carbon bonds, blue bonds, polar bonds, white bonds, sulphur-sulphur bonds.

The trend for Hb levels was the same; the highest dose (G5) had higher Hb (15.34 ± 0.36 g/dl) than the control (13.82 ± 0.50 g/dl), most likely because of the high iron content of chlorella, which is necessary for hemoglobin production. These results are consistent with those (Morita

et al. 1999), who found that the high iron and vitamin B_{12} content of chlorella increases hemoglobin synthesis.

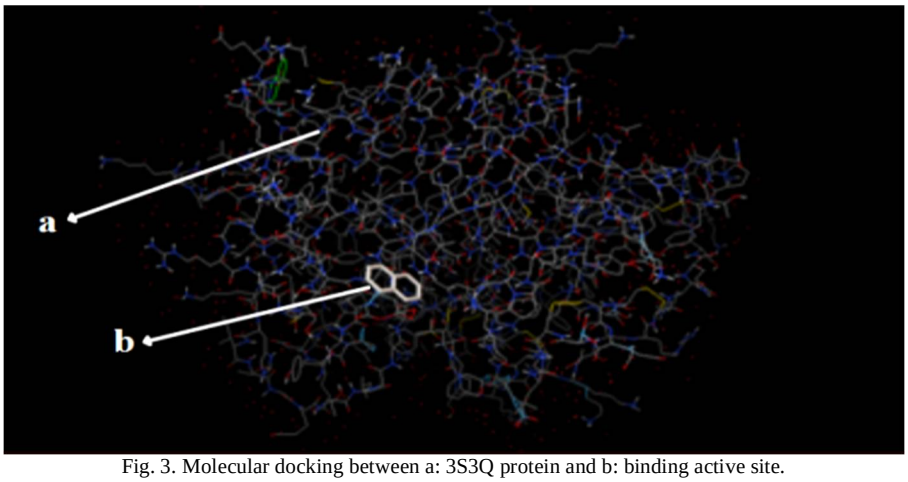

Fig. 3. Molecular docking between a: 3S3Q protein and b: binding active site.

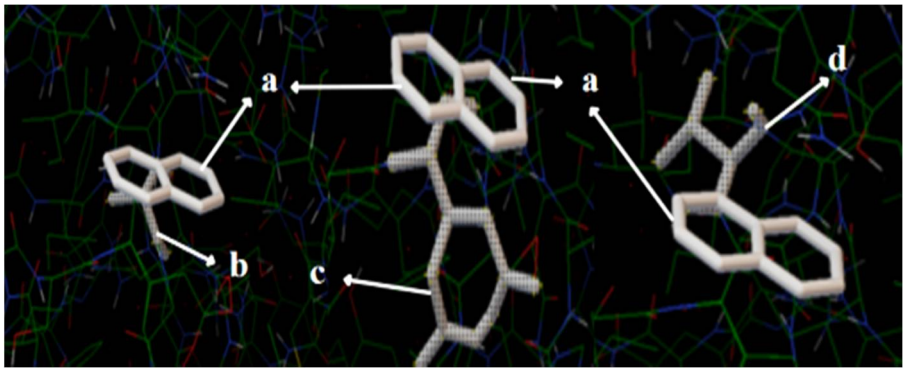

Fig. 4. Molecular docking between a: 3S3Q protein at b: Alanine 243, c: Glutamic acid 316, d: Valine 317 residues of active site.

Chicken blood cells that recognize and phagocytose infections include monocytes, thrombocytes, basophils, and heterophils (which are similar to mammalian neutrophils) (Dorhoi *et al.* 2006). This is consistent with earlier research demonstrating that *Chlorella vulgaris'* immunomodulatory bioactives, such as beta-glucans and polysaccharides, promote leukocyte proliferation (Kotrbáček *et al.* 2013, Bito *et al.* 2020). With G5 reaching $26.50 \pm 0.80\%$ compared to $22.50 \pm 1.80\%$ in the control, chlorella supplementation dramatically raised neutrophil percentages, suggesting an improved innate immune response. It is reported by Chen *et al.* (2021), that at lower dosages, the immunostimulatory effects of chlorella might promote lymphocyte proliferation, whereas at greater doses, the decrease would be a regulatory reaction to immunological overstimulation. The effects of varying dosages of *C. vulgaris* on oxidative stress indicators, including as MDA, PCO, TAC, SOD, CAT, and GSH, were evaluated in this study. The findings demonstrated that chlorella considerably lowered oxidative stress and improved antioxidant defenses. Poor physiological function is associated with elevated levels of MDA and PCO, two important markers of oxidative damage (Agarwal and Allamaneni 2004).

In comparison to the *Chlorella*-supplemented groups, MDA, a measure of lipid peroxidation, was considerably greater in the control group (23.75 ± 1.65 nmol/ml) ($p = 0.01$), with G5 exhibiting the largest decrease (14.90 ± 0.90 nmol/ml). This dose-dependent decline is consistent with other research showing the antioxidant capabilities of *C. vulgaris* in scavenging free radicals (Guzman *et al.* 2001, Papadaki *et al.* 2024). G1 (5.95 ± 0.24) and G5 (6.15 ± 0.31) had the lowest levels of PCO, a measure of protein oxidation, which was considerably greater in the control group (7.70 ± 0.56 µmol/ml) than in the Chlorella-treated groups ($p = 0.001$). These decreases imply that *C. vulgaris*, which is abundant in carotenoids, chlorophyll, and polyphenols, efficiently prevents oxidative damage to proteins (Schwerin *et al.* 2009).

SOD activity significantly increased with *C. vulgaris* supplementation ($p = 0.04$), with G5 showing the highest level (210.5 ± 4.00 U/ml), indicating strong enhancement of antioxidant defense at the highest dose. According to (Mirzaie *et al.* 2020), *C. vulgaris* strengthened antioxidant defenses by increasing SOD activity. Although there was a modest rise in CAT activity, it was not statistically significant ($p = 0.1$), indicating a less significant effect than that of SOD and GSH. This is in line with other research on the varying CAT responses to algal supplementation (Chen *et al.* 2021). By removing important food components' epoxide substrate inhibitors, the 3S3Q protein improves nutrient metabolism (Saleem *et al.* 2024). There is no considerable isoquinoline found in *C. vulgaris* naturally (Panahi *et al.* 2016). Only thermochemical procedures like hydrothermal liquefaction or pressurised pyrolysis may create isoquinoline from algal biomass. Therefore, rather than isoquinoline, the inherent nutritive and bioactive components of *C. vulgaris* are responsible for the results seen in hens. Isoquinoline alkaloid binding increases feed efficiency, improves nutrient absorption, lowers the risk of digestive injury, and increases gastrointestinal juice activity.

The study's findings show that supplementing chickens with *C. vulgaris*, particularly at higher doses, considerably lowers oxidative stress and improves their antioxidant defense systems. *Chlorella vulgaris* is able to successfully prevent oxidative damage based on the rise in TAC, SOD, and GSH and the decrease in MDA and PCO levels, potentially enhancing general health and stress resilience. These results are in line with other single cell green microalga belonging to the family Chlorophyta like *Chlamydomonas reinhardtii* (Abideen *et al*. 2025), *Haematococcus pluvialis* (He *et al*. 2023) and *Parietochloris incise* (Das and Roy 2025).

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