Egypt. J. Chem. **Vol. 68**, No. 5 pp. 515 - 526 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

The Role of *Nano-Spirulina platensis* in Enhancement of Female Rabbit Puberty and Amelioration of the Heat Stress Adverse Effect

Emtenan M Hanafi[1], Walaa H. Khalifa[2*],Elnady I.A.[3] Noura H.G [4], Enas N. Danial[5], Manal M Ramadan[6], Omima H.Ezzo[1], Amal M. Aboelmaaty[1], Abd El -Nasser A. Mdboli[1]

1 Department of Animal Reproduction and A.L., Veterinary Research Institute, NRC, Giza, Egypt.
2 Department of Animal Production, Agriculture and Biological Research Institute. NRC, Giza, Egypt.
3 Department of Animal Production, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.
4Department of Animal and Poultry Production Faculty of Technology and Development, Zagazig University.
5 Department of Chemistry of Natural and Microbial Product, NRC, Giza, Egypt.
6 Department of Flavour Aromatic Plants NRC, Giza, Egypt

Abstract

Spirulina platensis alga is an extreme protein source that enhances ovarian function and diminishes heat stress impacts. This work aims to study the enhancement effect of Nano-Spirulina-platensis alga on female rabbit puberty and its ameliorative effect against adverse effects of heat stress (HS). HS impairs growth and delaysthe puberty of rabbits. Spirulina-nanoparticles were prepared then; HPLC, antioxidant DPPH, total phenolic,and flavonoids were estimated. 24 New Zealand female rabbits were divided into 4 groups (n=6). G1 negativecontrol group under ambient temperature. G2 was exposed to heat stress (35°C). G3 and G4 were dietary supplied with Nano-Spirulina (15mg and 35mg/Kg bwt/30 days under heat stress respectively. Rabbits body temperature, respiration rate, body weight gain, feed intake, food conversion rate (FCR), feed efficiency ratio (FER), and puberty behavior observation were recorded. Blood plasma was collected and analyzed for thereproductive hormones; follicle-stimulating hormone(FSH), luteinizing hormone(LH), and progesterone(P4).Also the proliferative marker Transforming-Growth-Factor-Beta-2 (TGFβ2), and the cytokines as; Gamma-Interferon(INFγ), Interlukine-1(IL-1), and Tumor-Necrosis-Factor α(TNFα). In addition to the liver and renalfunction markers Albumin, Alanine-Transaminase (ALT), Aspartate-aminotransferase(AST), and Creatinine.Also, the oxidative stress marker Malondialdehyde (MDA) and antioxidant markers Catalase and Superoxide-Dismutase(SOD) were measured. Finally; rabbits were slaughtered and histopathological investigations wereperformed for the ovary, small intestine, liver, and spleen. Our findings showed that; Nano-Spirulina improvedthe oxidative status, elevated the reproductive hormones, and protected the examined tissues from heat stresseffects. Spirulina stimulated folliculogenesis earlier than control rabbits. It could be concluded that; Nano-Spirulina enhanced puberty in female rabbits during heat stress.

Keywords: Nano-Spirulina; puberty; heat stress; ovary; rabbit.

Introduction

Rabbit puberty was reported to be delayed as the age at first mating increased with elevated environmental temperature and humidity. Egyptian native breeds reached puberty at about 6 months of age this may be due to the negative effect of heat stress on feed intake and body weight gain. The enforced mating practice resulted in very low conception rate and an increase in neonatal loss [1]. Plenty of nutritional studies were done to improve female reproductive traits like ovarian follicular activity and conception rate. Spirulina platensis is emerging as a potential candidate to meet these criteria. It is rich in vitamins, minerals, antioxidants, and phenolic compounds [2]. It was reported that; when Spirulina platensis was fed to animals, it showed a high feed conversion rate and optimized animal product quality and quantity. Dietary Spirulina had immunogenic activity, increased the natural killer cell activity (NK), and enhanced the disease resistance [3]. A previous study reported that administration of Spirulina platensis stimulated sex hormones and enhanced ovarian function and follicle formation in females [4]. It is well-known that nanoparticles are more targeted, can persist in the bloodstream for a longer time, and give a more

*Corresponding author e-mail: walaahussein10@gmail.com
Receive Date: 01 September 2024, Revise Date: 25 September 2024, Accept Date: 29 September 2024
DOI: 10.21608/ejchem.2024.316799.10302

favorable effect than conventional particles [5]. The current study aimed to explore the effect of supplementing different doses of Nano-Spirulina on the general health of female rabbits with special emphasis on the onset of puberty.

2. Materials & Methods

2.1. Identification of active component of the microalgae

The microalgae were provided by the Alga Biotechnology Unit/National Research Center (NRC)/ Dokki/Egypt. Nutritional analysis and active composition were identified by atomic absorption and HPLC units in NRC according to [6].

2.2. Preparation of the nano particles

The particle size of dried algae was reduced by mechanical sonication. The z-average hydrodynamic diameter was determined, in study of samples at 25°C by photon correlation spectroscopy using a Zeta Sizer Nano (ZS) (Malvern Instruments Inc., Southborough, MA) in the Central Lab in NRC).

2.2.1. Morphology of nanoparticles using Transmission Electron Microscope (TEM)

The examined samples' z-average hydrodynamic diameter was estimated at 25 ± 0.1°C by photon correlation spectroscopy using a Zeta Sizer Nano ZS (Malvern Instruments Inc., Southborough, MA). Morphology of sprayed powder was done using TEM.

2.2.2. Cytotoxic effect of Nano-Spirulina on human normal fibroblast cell line (BJ1)

Cell viability was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan [7].

.2.3. Estimation of Nano-Spirulina total phenolic and flavonoid contents

The total phenolic and flavonoid content was determined using gallic acid and rutin as standard [8-9].

2.4. Determination of antioxidant activity of Nano-Spirulina

The free radical scavenging capacity of microalgae was determined using the stable 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) compared to ascorbic acid as standard [10].

2.5. Polyphenol profile of Nano-Spirulina by HPLC:

Polyphenols HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using the Eclipse C18 column (4.6 mm x 250 mm i.d., 5 μm) [11].

2.6. Biological study:

2.6.1. Ethics:

The biological experiment was designed and done under the regulation of the ethical committee of NRC (project no.13050419). Female rabbits were kept in clean well-aerated chambers and had free access to feed and water. 12 hours light period. Kept in a naturally ventilated and lighted rabbitry. Rabbits were housed individually in stainless steel cage batteries (40 × 50 × 35 cm) supplied with feeders and automatic fresh-water nipples.

2.6.2. Experimental design

A total number of 24 Healthy New Zealand white female rabbits 2 months old weighing 1.5 kg bwt were used in this study. The experimental rabbits were kept in a temperature-controlled room at 35°C to keep rabbits under heat stress (HS) except the control group and were fed on a commercial pelleted diet (Table 1) cover in their daily nutritional requirements [12]. Rabbits were equally divided into four groups (n=6). G1 was fed only on the basal diet and kept at ambient temperature. G2 was fed on the basal diet and kept under HS. G3 and G4were fed on the same diet besides oral administration with Spirulina platensis nanoparticles (15mg and 35mg /Kg bwt respectively) and kept under heat stress. Rabbits' body temperature, body weight gain, feed intake, feed conversion rate (FCR), feed efficiency ratio (FER), and vitality of rabbits were recorded. The treatment period was 30 days. Females were observed for the onset of puberty behavior.

2.6.3. Heat stress room environmental condition

The heat stress room was equipped with thermometers and humidity-measuring equipment, the minimum and maximum average temperatures were measured and recorded in Table (2). The Average temperature-humidity

index (THI) was predestined regularly based on [13] as follows: THI = Tdb – (0.31 - 0.31 RH) (Tdb - 14.4).Where Tdb presents the dry bulb temperature in Celsius; RH presents the relative humidity percentage. The corresponding stress levels for the following THI values;27.8, 27.8 to 28.9, 28.9 to 30.0, and ≥30 were identified as no stress, moderate, severe, and very severe heat stress, respectively. PAs it is clear from the table that; animals suffer from severe to very severe heat stress.

Table 1: Formulation of basal diet

Ingredient	**%**	**Chemicalanalysis**	**%**
Berseemhay	30.0	Drymatter(DM)	87.7
Barleygrain	24.6	Organicmatter(OM)	88.9
Wheatbrain	21.5	Crudeprotein(CP)	17.8
Soybean(44% CP)	17.5	Crudefiber(CF)	14
Molasses	3.0	Etherextract(EE)	2.23
Limestone	1.0	Nitrogenfreeextract(NFE)	59.5
Di-calciumphosphate	1.6	Ash	8.6
Sodiumchloride	0.3	Metabolizableenergy(ME,kcal/kg)2	23.14
Mineral–vitaminpremix1	0.3	Calcium2	1.24
Dl-Methionine	0.2	Phosphorus2	0.81
Total	100	Methionine2	0.45

Table 2. Ambient temperature (C°) relative humidity (RH%) and THI values in different weeks of the experimental period under heat stress

	Heat stress conditions for G2, G3 and G4					
Week	**C°**		**RH%**		**THI**	
	Minimum	**Maximum**	**Minimum**	**Maximum**	**Minimum**	**Maximum**
1	19.29±0.42	35.14±0.5	22.86±2.20	61.86±1.4	29.38±0.55	32.51±0.55
2	19.86±0.96	40.86±1.79	24.57±3.83	56.43±1.97	28.36±1.54	37.06±1.54
3	18.86±0.26	36.29±0.89	31.43±4.63	72.71±3.51	28.65±0.72	32.38±0.72
4	17.14±0.34	34.86±0.26	31.29±0.28	56.86±2.25	30.14±0.16	31.08±0.16
	Normal conditions for G1					
	Minimum	**Maximum**	**Minimum**	**Maximum**	**Minimum**	**Maximum**
1	19.4±0.44	29.14±0.52	35.86±2.20	61.86±1.42	24.38±0.54	27.14±0.57
2	21.86±0.85	29.86±1.75	32.57±3.81	68.43±1.95	23.94±1.57	27.45±1.52
3	17.86±0.24	29.29±0.86	32.43±4.62	74.71±3.54	26.39±0.70	27.83±0.73
4	17.14±0.32	29.86±0.27	32.29±0.27	68.86±2.26	26.44±0.14	27.14±0.15

2.6.4. Plasma biochemistry

At the end of the experiment, animals were slaughtered and blood was collected. Plasma chemistry was analyzed for the reproductive hormones Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), and progesterone (P4). Also measuring of the proliferative marker Transforming-Growth-Factor-β-2 (TGFβ2),and some cytokines such as Gamma-Interferon (INFγ), Interlukine-1 β (IL-1β), and Tumor-Necrosis-Factor α (TNFα). In addition to; the liver and renal function markers as Albumin, Alanine-Transaminase (ALT),Aspartate-aminotransferase (AST), and Creatinine. Also, measuring the oxidative stress marker Malondialdehyde (MDA) and antioxidant markers Catalase and Superoxide-dismutase (SOD). These parameters were measured by using an ELISA commercial kit (SUNLONG, China). The sensitivity of the assay was 0.05ng/ml for rabbit P4, 0.1ng for FSH, 0.01 ng/ml for LH, 0.5 pg/ml for INFϒ, 0.1pg/ml for TNFα,0.6 pg/ml for TGFB2, and 0.7 pg/ml for IL-1β. The intra and inter-assay precisions for all the ELISA kits were 10% and 12%. The total protein, albumin, cholesterol, ALT, AST, creatinine, MDA, SOD, and catalase were analyzed using colorimetric diagnostic kits (Biodiagnostic, Egypt).

2.6.5. Histopathological Examination

At the end of the experiment, a histopathological examination was carried out for all experimental rabbits. Tissue specimens from the ovary, small intestine, liver, and spleen were taken to report the histo pathologica lchanges in these tissues. Tissue specimens are fixed at 10% neutral buffered formalin (NBF). Routine tissue processing was performed as dehydration, embedding in paraffin wax, and sectioning at 3–5 μm. The obtained tissue slides are stained with H&E for histopathological examination [14].

2.6.6. Statistical analysis

Data were analyzed by one-way ANOVA using SPSS 20.0 [15]. If a significant difference ($P \leq 0.05$) was detected, Duncan's test was performed for multiple comparisons [16]

3. Results & Discussion:

3.1 Analysis of nutrients of microalgae

Analysis of Nano-Spirulina (Table 3, 4) showed that Nano-Spirulina is rich in protein,carbohydrate, phosphorus, calcium, magnesium, iron, zinc, selenium, copper, and vitamins A, D3, and E. These findings were clarified by a previous study that reported a high nutritive value of Spirulina platensis[17].

Table3: Analysis of NanoSpirulina nutrients and vitamins

Chemistry	**(% of dry matter basis)**
Carbohydrate protein	39.92
Fat Ash	56.2
Fiber	0.72
Dry matter Organic	1.9
matter Vitamins	1.26
Vit A VitD3	90.45
Vit E	98.1
	(IU)
	4550
	1427
	58

Table 4: Estimation of mineral and traces in nanospirulina

Minerals and traces	**mg/kg**
Phosphorus Calcium	3500
Magnesium Iron	1875
Selenium Copper	1050
zinc	220
	1.25
	1.5
	14.5

3.2. Morphology of nanoparticles using Transmission Electron Microscope (TEM)

The morphology and size of the nanoparticles were estimated by transmission electron microscope which was33 nm (Fig1). The physical stability of the particles depends on the size of a colloidal system's ζ-potential(Zeta potential: which is an electro-kinetic potentiality for the particles in colloidal dispersions).If all of the particles had strong ζ-potential (either positive or negative), they repel each other and did not form aggregations [18]. The ζ-potential value was 30.7 mV (millivolts) in Spirulina which indicated repulsive interactions between charged molecules as if the ζ-potential of the particles were greater than 30 mV they are physically constant [19-21]

3.3. Cytotoxic effect of Nano-Spirulina on human fibroblast cell line (BJ1)

The present study revealed that; 100 ug/ml or ppm conc. of the Nano-Spirulina killed 13.5% of the normal human epithelial cell line BJ1 (normal skin fibroblast) and this is the normal physiological range. That means; the Nano-Spirulina has a great biosafety on somatic cells [22].

3.4. Estimation of the total phenolic and flavonoid contents in the Nano-Spirulina alga

The illustrated findings in Fig (2) exhibited that; the Nano-Spirulina extract is rich in flavonoids (3.9) asmatched to gallic acid (2.59), rutin (2.7). In addition, the estimated total phenolics is (36.9) as matched to gallicacid (36.5) and rutin (40) All these records were standardly expressed as mg/gm dry weight [23].

3.5. Antioxidant activity

The antioxidant activity of the Nano-Spirulina extract which is estimated in Fig (3) has a high antioxidant activity (DPPH) as compared with ascorbic acid.This finding could be clarified as, Spirulina is rich in vitamins, and

minerals (Table 4) and also gallic acid, andferulic acid (Table 5) these components the strong antioxidant activity as confirmed by Enyidi [24].

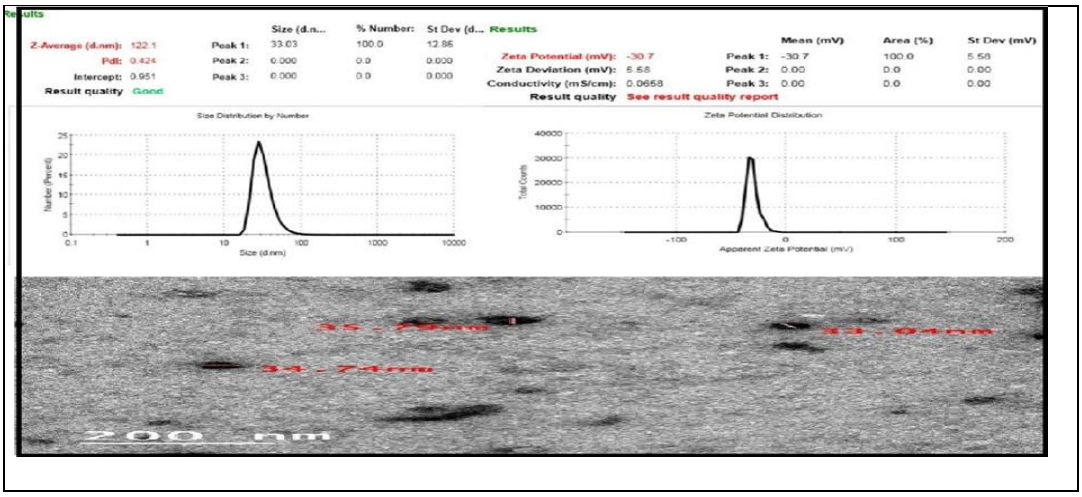

Fig 1: Zeta sizer and morphology of nano particles using TEM

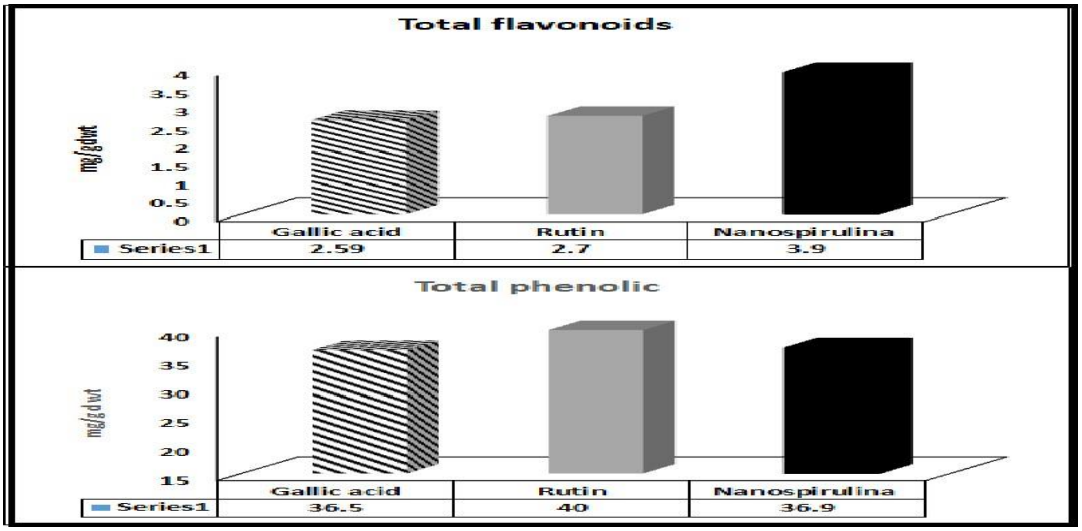

Fig 2. Total flavonoid, total phenolic content of Nano-Spirulina extract

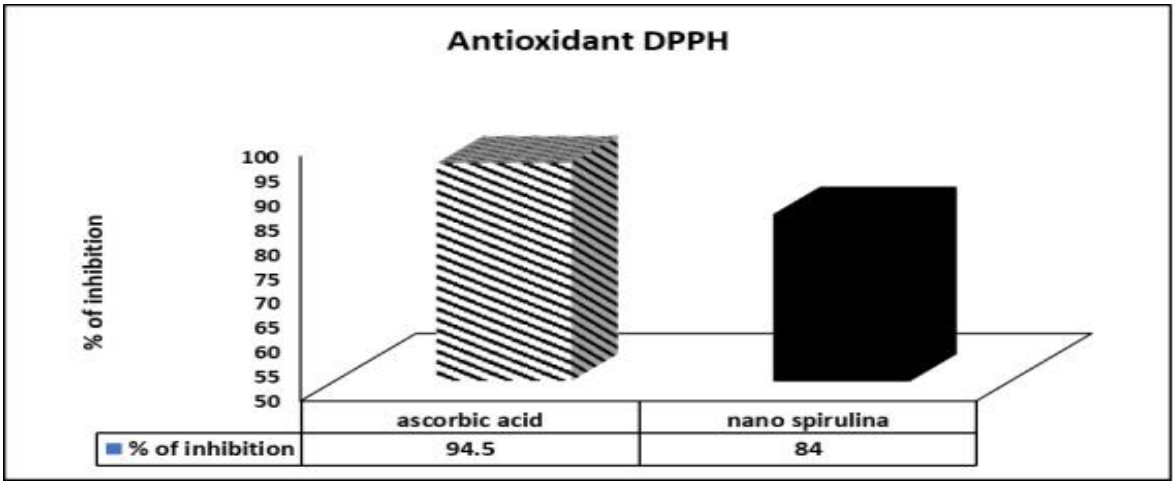

Fig 3. Antioxidant activity (DPPH) of Nano-Spirulina extract

3.6. Polyphenol profile (HPLC)

The screening process of the polyphenolic compounds of the Nano-Spirulina extract using HPLC anddisplayed in Table (5) showed that; Gallic acid is the dominant compound which is previously reported and well established to be a strong antioxidant [25]

Table 5: Polyphenol profile (HPLC) of Nano spirulina extract

Polyphenol compound	Spirulina platensis (ug/ml)	Slandered conc.(ug/ml)
Gallic acid	9.40	8.4
Coffeic acid	0.25	9
Ferulic acid	0.20	6.0
Naringenine	0.21	7.5
querectin	0.34	6.1
Cinnamic acid	0.09	2.3
Kaempferol	0.34	6
Chlorogenic acid	ND	14
Rutin	ND	30.5
Daidazin	ND	10
Vanillin	ND	6.15
Ellagic acid	ND	17.15

3.7. Growth performance

The growth performance of the present work as in Fig (4) revealed that; animals kept under heat stress (G2) showed a decrease in both feed intake, body weight gain, and feed efficiency ratio, in addition to thehistopathological picture of the small intestine in fig. (6) Which showed a destruction and degeneration of the cell lining of intestinal that leads to the limitation of the nutrient absorption in this group.Administration of the Nano-Spirulina led to an improvement in the parameters of feed intake, body weight gain, and Feed Efficiency Ratio (FER), especially at (G3) (15 mg/kg bwt) which recorded the highest values of these parameters. These results were supported by our histological findings of the intestinal villi (Fig 6).These findings illustrated the reverse effect that was clarified in (G3) and (G4) due to the antioxidant effect of Nano Spirulina. Spirulina was riched in phenolic compounds that have antioxidant effects [25] and so lowered the animals; body temperature and enhanced the antimicrobial activity which leads to clearing of the alimentary tract from pathogens therefore increasing the nutrient absorption and feed utilization. It is also rich in protein,minerals, and trace elements necessary for anabolism [26].

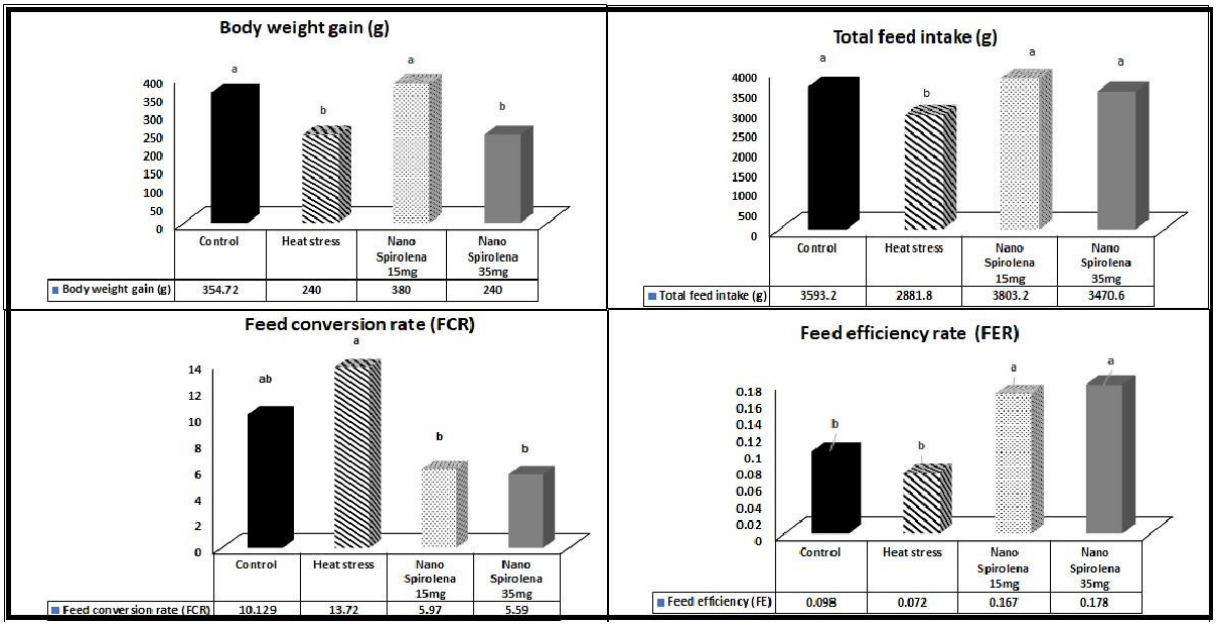

Fig 4: Effect of using nano spirulina on growth performance of rabbits.

3.8 Plasma chemistry

The analytical findings of plasma chemistry in the current study displayed in Table (6) showed that; Rabbitskept under heat stress recorded low levels of both antioxidant markers (SOD and Catalase), LH hormone, and TGFβ 2 . Also recorded a high levels of liver and kidney function markers (ALT, AST, and creatinine), oxidative markers (MDA), and cytokines (INFγ) as in comparison with G1 (the control group kept under ambient temperature). These results were supported by our histopathological findings (Fig 7) that showed degenerative changes and periportal congestion among hepatic cords in the liver of G2, subjected to heat stress

These records could be explained as; Heat stress is associated with oxidative stress and elevated inflammatory cytokines [27] and is well known to harm all the body's metabolic processes, showing pathological changes inthe liver, kidney, and ovary [28].On the other side; the results of the administration of Nano-Spirulina in two levels exhibited in Table (6)showed that; the examined plasma chemistry of rabbits in G3 & G4 recorded high levels of the antioxidant enzymes (Catalase and SOD) as well as high levels of the reproductive hormones FSH, LH, and progesterone indicating early puberty. In addition; administration of the Nano-Spirulina slightly elevated ALT, AST, andcreatinine, and also elevated IL-1 β and INFγ. Moreover, the histological findings of these two groups of the liver did not show any pathological changes and showed complete protection for tissues due to theadministration of Nano-Spirulina. Also, Fig (7) showed the normal hepato-portal area with normal healthy hepatic cords [29].

Table 6: Effect of administration of Nano-Spirulina on plasma chemistry of rabbits

	Control	Heat stress	*Spirulina 1*	*Spirulina 2*	P value
Total protein (g/dl)	3.13 + 0.20^{a}	3.19 +0.38^{a}	3.03+0.31^{a}	3.62+0.17^{a}	0.400
Albumin (g/dl)	2.51+ 0.09^{a}	2.99 + 0.39 ^{a}	2.13 + 0.65^{ab}	1.95 +0.38^{b}	0.011
Globulin (g/dl)	0.62+0.23a	0.57 + 2.06a	1.26 + 1.12b	1.66+0.49b	0.508
Cholesterol (mg/dl)	44.79+15.8a	33.42 + 2.06b	29.32 +2.15b	38.51+2.15b	0.613
Creatinine (mg/dl)	0.52 +0.21^{a}	1.34 +0.12^{b}	1.63 +0.47^{b}	0.71 + 0.25^{a}	0.000
AST (U/L)	14.56+1.04^{a}	24.9 + 8.56^{b}	26.31 +4.54^{b}	20.13 + 6.07^{ab}	0.034
ALT (U/L)	3.82 +0.31a	6.81+2.27b	7.6 + 4.47b	8.62 +5.17b	0.321
Catalase(nmol/ml)	346.15+56.84^{a}	180.70+ 60.94^{b}	247.60+17.12^{a}	248.37+ 27.88^{a}	0.000
SOD (U/ml)	287.5+80.6^{a}	137.5 + 48.7^{b}	250.0 +51.03^{a}	291.6+ 41.6^{a}	0.006
MDA(nmol/ml)	7.76 + 0.68^{a}	9.31+ 0.99^{b}	7.05 + 0.35^{a}	7.28 + 0.81^{a}	0.000
P4 (ng/ml)	60.75 + 0.19^{a}	60.75 + 0.15^{a}	50.87 + 0.14^{b}	50.08 + 0.23^{b}	0.533
LH (ng/ml)	10.26 + 0.05^{a}	10.19 + 0.01^{a}	10.49 + 0.01^{a}	8.49 + 0.09^{b}	0.000
FSH (ng/ml)	31.47 + 0.14^{a}	26.95 + 0.41^{b}	38.45 + 23.3^{c}	35.30+4.28^{c}	0.000
IL-1β (pg/ml)	400.04 + 0.47^{a}	450.07 + 0.45^{ab}	560.84 +1.05^{b}	569.71 +0.32^{b}	0.000
TGFβ$_2$ (pg/ml)	145.92 +3.51^{a}	113.60 +2.22^{c}	116.07 + 4.62^{b}	114.90+0.57^{c}	0.000
IF-γ (pg/ml)	92.83 +1.05^{a}	80.48+1.91^{b}	140.48 +3.38^{c}	150.0 +7.73^{d}	0.000
TNF-α (pg/m)	21.94 + 0.31^{a}	21.25 +0.09^{a}	18.58 + 0.44^{b}	15.62 +1.06^{c}	0.000

Data were presented as Means + SE. Different superscripts within row means significance (P ≥ 0.05).

Spirulina is an immunomodulator. It acts on the receptors of the natural killer cells (NK) and induces up-regulation of some cytokines (IL-1β and INFγ) while other cytokines are down-regulated like TNFα and TGFβ. Spirulina is rich in vitamins and minerals as shown in Table (5) so it has high antioxidant activity as mentioned in Fig (3) and is rich in flavonoids and phenolic compounds as mentioned in Fig (2) and this was reflected in plasma chemistry. Similar results in rabbits fed spirulina were recorded [30]. The histological picture of the ovary (Fig 5) confirmed the hormone profile as the elevated FSH and LH expressed as multiple mature follicles and corpus luteum indicating ovulation and early puberty as compared to control animals. Previous studies reported that; the administration of an aqueous extract of Spirulina platensis affected the reproductive performance of rabbit doe and recorded elevated LH, estradiol, and serum protein concentration [31].

3.9. Histopathological study

The current histopathological study was performed to shed light on the relationship between heat stress and the detected lesions in ovaries, intestine, liver, and spleen.

3.9.1 Ovary

The ovarian tissue of the control rabbits (G1) contained moderate numbers of the different follicular stages as primordial and mature follicles (Fig. 5A). On the other hand; The prominent changes in ovaries of the heat stress-

exposed immature rabbit doe (G2) were shown asinadequate ovarian follicular activity which was exhibited in the form of small numbers of primordial follicles and a great number of atretic follicles (Fig. 5B).

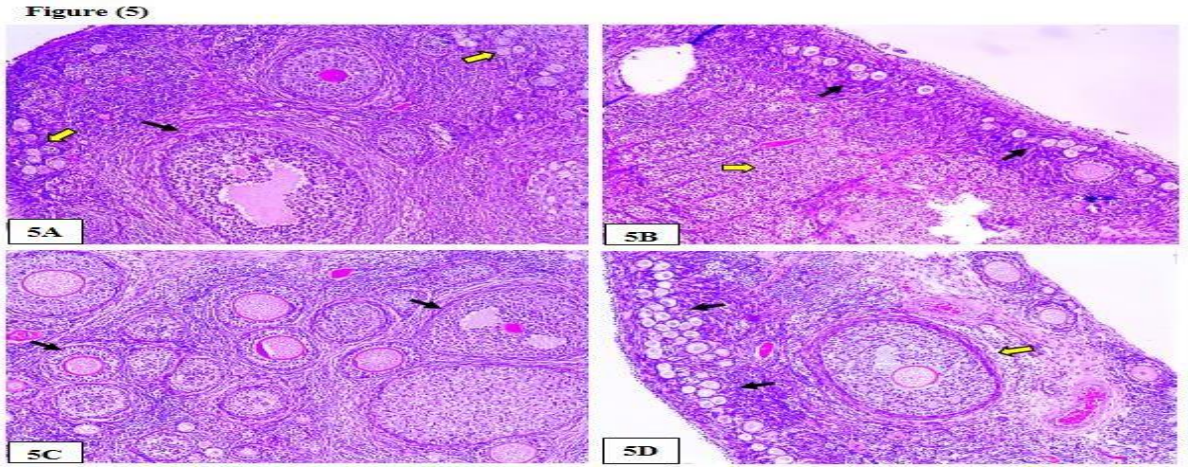

Figure (5): The histopathological changes in the ovary of the four experimental groups:

Fig. 5A: Ovary of the rabbit doe showed normal tissue architecture containing moderate numbers of the different stages of folliculogenesis as mature follicles (black arrow) and primordial follicles (yellow arrows). Fig. 5B: Ovary showed poor follicular activity characterized by the existence of small primordial follicles (black arrows) associated with the existence of large numbers of atretic follicles (yellow arrow). H&E X 100. Fig. 5C: Ovary of (G3) showed an FSH-like effect for the Nano- Spirulina Platensis algae as the existence of the ovarian follicles in different stages with large numbers adjacent to each other (arrows) H&E X 100. Fig. 5D: Ovary of (G4) showed a moderate FSH-like effect for the Nano-Spirolina Platensis algae which exhibited the existence of moderate numbers of primordial follicles (black arrows) in addition to a few mature follicles (yellow arrow) H&E X 100.

It was reported that; thermal stress causes different changes in the tissue details of several organs [32]. Heat stress causes an elevation in the production of transition metal ions, which can induce electron donations to oxygen-constituting superoxide (H2O2) which is afterward reduced to reactive oxygen species (ROS) leading to oxidative stress [33], which consequently leads to metabolic and functional disorders in various cells and directly affects the growth, immunological state, and reproductive efficiency of animals [34]. These stressors effects expressed as; ovarian tissue changes, follicular degeneration or atresia, and lysis of oocytes [32].The Nano-Spirulina Platensis-treated rabbit doe (G3 and G4) results clarified the safeness effects of Spirulina algae on the rabbit doe tissues and in addition; repulsed the heat stress effects on the ovarian tissue and enhanced their follicular activity where it showed an increase in the follicular number and corpora lutea with a pronounced decline in the follicular atresia (as compared with the G2 kept under heat stress), especially in G3 (15 mg/kg Nano-Spirulina Platensis treated rabbits). (Fig. 5C). On the other hand, G4 (35mg/kg Nano-Spirulina Platensis-treated rabbit doe) exhibited the presence of moderate numbers of primordial follicles and a few mature follicles (Fig. 5D). These findings could be due to the Spirulina anti-inflammatory and antioxidant properties that hinder the oxidation process and ameliorate the cell injury [35].

3.9.2. Small intestine

The small intestine of the control rabbit doe (G1) showed normal intestinal villi with intact columnar epithelium and normal tissue cores (Fig. 6A). The heat stress-exposed rabbit doe (G2) exhibited destruction of most of the intestinal villi associated with mild hyperplasia in the lining epithelium of the apical portion of some intestinal villi (Fig. 6B).

It was reported that; circulating cytokines may contribute to tissue damage. The changes in concentration of several cytokines and chemokines like GM-cSF, McP-1, MIP-1α, IL-4, IL-1β, Il-2, IL-6, IL-10, IL-12p40, and TNF-α produced in the ileum tissue that induces histopathological changes. If IL-1βis in high levels in the small intestine, it plays an antimicrobial role in protecting the intestinal mucosa, but the overdose of this cytokine causes injury to the intestinal epithelium leading to apoptosis and necrosis [36]. Also, a positive correlation was found between intestinal IL-10, which was elevated during heat stress, and tissue injury [37]. Results of the current study showed that administration of15mgNano-Spirulina platensis to the heat-stressed rabbit doe (G3) illustrated histologically normal intestinal villi lined with healthy intact simple columnar epithelium with active

goblet cells, (Fig. 6C). In addition; the35mgNano-Spirulina platensis-treated group (G4) showed the same picture associated with mild necrosis in the lining epithelium of the apical portion of the intestinal villi (Fig. 6D). These results could be attributed to the antioxidant and anti-inflammatory effects of Nano-Spirulina. Also, these histopathological findings were confirmed by our finding in Table (4) where the cytokines level was lower in Nano-Spirulina-treated groups as compared with **other groups [38].**

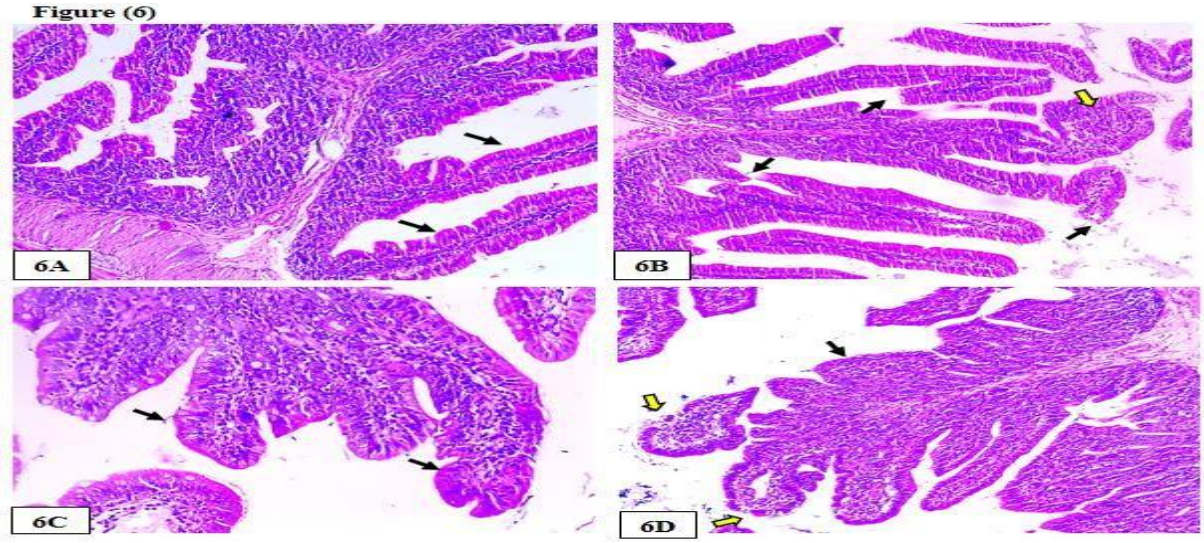

Figure (6): The histopathological changes in the intestine of the four experimental groups

Fig. 6A: Intestine illustrated normal intestinal villi with normal height and intact columnar epithelium with normal tissue cores (arrows). Fig. 6B: Intestine showed destruction of most of the intestinal villi (black arrows) associated with hyperplasia in the lining epithelium of the apical portion of some of the intestinal villi (yellow arrow). H&E X 100. Fig. 6C: Intestine exhibited healthy intact intestinal villi lined with normal intact simple columnar epithelium with active goblet cells, (arrows) H&E X 200. Fig. 6D: Intestine showed healthy intestinal villi lined with normal intact simple columnar epithelium (black arrows). Still, mild necrosis was noticed in the lining epithelium of the apical portion of the intestinal villi (yellow arrows) H&E X 200.

3.9.3. Liver

The hepatic tissue of the control rabbit doe (G1) was illustrated as normal healthy hepatocytes with a noncongested normal portal triad (Fig. 7A). In the heat stress-exposed rabbit doe (G2), periportal hemorrhage adjacent to the bile ductules accompanied by mild congestion among the hepatic cords was observed (Fig. 7B).

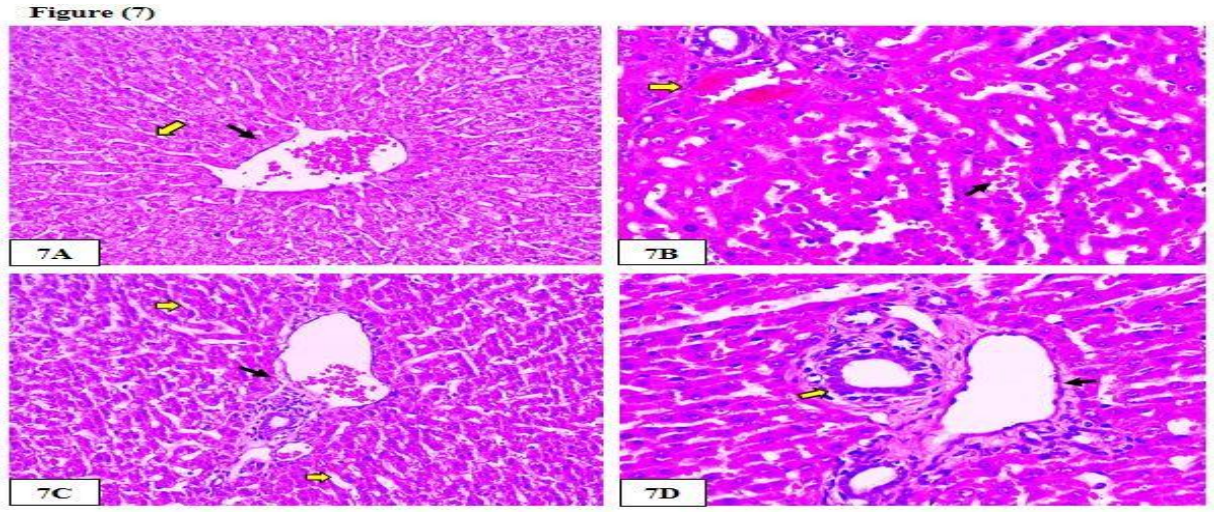

Figure (7): The histopathological changes in the liver of the four experimental groups:

Fig. 7A: The Liver exhibited normal non congested non-dilated central veins (black arrow) and normal hepatic cords (yellow arrow).Fig. 7B: The liver illustrates periportal hemorrhage adjacent to the bile canaliculi (yellow arrow), which is accompanied by hemorrhages among the hepatic cords (black arrow). H&E X 200. Fig. 7C: Liver illustrated normal hepato-portal area with the normal non-congested portal vein (black arrow) Surrounded with normal healthy hepatic cords (yellow arrows).Fig. 7D: Liver displayed histologically normal hepato-portal area with the normal non-congested portal vein (black arrow) Surrounded with normal healthy hepatic cords and normal intact bile ductules (yellow arrow) H&E X 400.

It was reported that; the thermal insult causes different histopathological changes in several organs as a result of elevated free radicals causing tissue injury [32].Previous study mentioned that; hepatic tissue was highly sensitive to heat stress which induced high portal venous radical content, leading to hepatocyte hypoxia [28]. Another study showed hepatic vessel congestion, perivascular leucocytic infiltration, and hepatic degeneration with focal necrosis in heat-stress exposed liver [39 and 40]. The two groups (G3 and G4) of rabbits given Nano-Spirulina platensis (Fig. 7C&7D) showed a normal hepato-portal area with a normal noncongested portal vein surrounded by normal healthy hepatic cords. This amelioration in the tissue architecture may be referred to the antioxidant and anti-inflammatory effect of Spirulina [33]

3.9.4. Spleen

The splenic tissue of the control rabbit doe (G1) displayed normal lymphoid follicles fully packed with lymphocytes and a normal splenic artery (Fig. 8A).

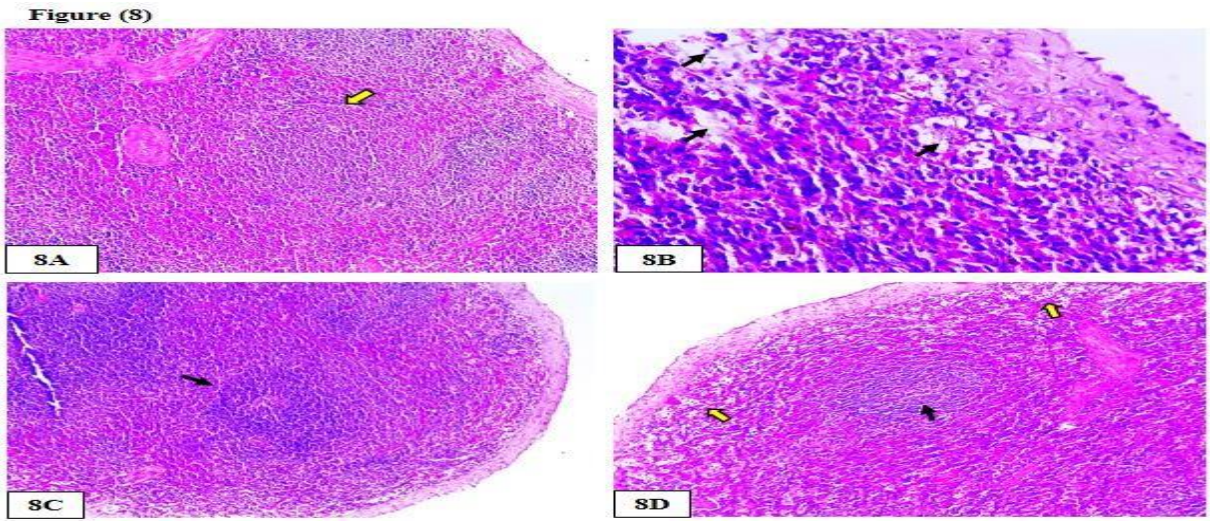

Figure (8): The histopathological changes in the spleen of the four experimental groups:

Fig. 8A: Spleen displayed normal active lymphoid follicles with normal splenic artery (arrow). All figures H&E X 100.Fig. 8B: Spleen displays lymphocytic necrosis with lymphocytic nuclear pyknosis leaving a white zone around cells. H&E X 200. Fig. 8C: Spleen displayed normal splenic tissue with normal lymphoid follicles in the white pulp (arrow). H&E X 100. Fig. 8D: Spleen illustrated normal lymphoid follicles in the white pulp (black arrow) but also, moderate subcapsular lymphocytic necrosis was seen (yellow arrow). H&E X 100.

In addition, the heat stress-exposed rabbit doe (G2) exhibited subcapsular lymphocytic necrosis (Fig. 8B). In (G3) the 15mg/kg Nano-Spirulina platensis-treated rabbit doe; normal lymphoid follicles in the white pulp (Fig. 8C). While; in the (G4) 35mg/kg Nano-Spirulina platensis-treated rabbit doe illustrated normal lymphoid follicles in the white pulp but also, moderate subcapsular lymphocytic necrosis was detected (Fig. 8D).The amelioration effects of Nano-Spirulina Platensis on splenic tissue could be related to that; Nano-spirulina has several health advantages on different body organs as a reduction in oxidative stress [27].

Conclusion

It can be concluded that the administration of Nano-Spirulina to young rabbits stimulates the growth rate, food efficiency ratio, improves ovarian function, and induces early puberty. Also, it improves the general condition of rabbits under heat stress and increases the animal vitality. It can be recommended that; dietary supplementation of growing animals with nano-particles of Spirulina as a small dose of Nano-Spirulina was more effective and targeted than ordinary micro size.

5. Acknowledgments
All work team would like to express their thanks for NRC Egypt which funded this work (project No 13050210).

6. Declaration of Conflict of Interest
The authors declare that there is no conflict of interest.

7. Ethical of approval
This study fulfilled the requirement and guidelines of good medical and laboratory practice (GCP and GLP) guidelines as well as institutional and animal care and use committee (LACUC) guidelines, recommendations, and rules regarding the ethics of scientific research and approved in July 2020.

8. References

[1]- A.F., Hilmy, Some productive aspects in rabbits, M.Sc. Thesis, Faculty of Agriculture Moshtohor Zagazig University Egypt (1991).
[2]-P., Howe, B., Meyer, S., Record, K., Baghurst, Dietary intake of long-chain omega-3 polyunsaturated fatty acids: contribution of meat sources, Nutrition, 22(1) (2006) 47-53.doi: 10.1016/j.nut.2005.05.009.
[3]-H.A., Al-Batshan, S.I., Al-Mufarrej, A.A., Al-Homaidan, M.A., Qureshi, Enhancement of chicken macrophage phagocytic function and nitric production by dietary Spirulina platensis. Immunopharmacology and Immunotoxicology 23 (2) (2001) 281-289.doi: 10.1081/iph-100103866.
[4]-S.A.E., Bashandy, S.A., El Awdan, H., Ebaid, I.M., Alhazza, Antioxidant potential of Spirulina platensis mitigates oxidative stress and reprotoxicity induced by sodium arsenite in male rats, Oxidative Medicine and Cellular Longevity, 1(2016)1–8. doi: 10.1155/2016/7174351
[5]-T., Niemiec, A., Łozicki, R., Pietrasik, S., Pawęta, A., Rygało-Galewska, M., Mtusiewicz, K., Zglińska, Impact of Ag nanoparticles (AgNPs) and mltimicrobial preparation (EM) on the carcass, mineral, and fatty acid composition of Cornu aspersum snails. Animals. 11(7) (2021) 1926. https://doi.org/10.3390/ani11071926
[6]- AOAC, Association of Official Analytical Chemists, Official Methods of Analysis, 21th ed Washington DC USA. (2019).
[7]-M.I., Thabrew, R.D., Hughes, I.G., Mcfarlane, Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. Journal of Pharmacy and Pharmacology, 49(11) 1132-1135 (1997).doi: 10.1111/j.2042-7158.1997.tb06055.x.
[8]-A., Kamboj, R., Gupta, A., Rana, R., Kaur, Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from extracts of terminalia bellerica. European Journal of Biomedical and Pharmaceutical Sciences, 2(3) (2015), 201-205.http://www.ejbps.com
[9]- E.A., Ainsworth, K.M., Gillespie, Estimation of total phenolic content and other oxidation substrates in plant tissues using folin-ciocalteu reagent Nature Protocols, 2 (4)(2007) 875-877.doi:10.1038/nprot.2007.102.
[10]- S., Baliyan, R., Mukherjee, A., Priyadarshini, A., Vibhuti, A., Gupta, R.P., Pandey, C.M., Chang, Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of Ficus religiosa. Molecules. 16; 27(4) (2022) 1326.doi: 10.3390/molecules27041326.
[11]-S., Tian, K., Nakamura, H., Kayahara, Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. Journal of Agriculture and Food Chemistry, 52(15) (2004), 4808-4813. https://doi.org/10.1021/jf049446f.
[12]-National Research Council, Nutrient Requirements of Poultry 9th Revised Ed (Washington, D.C. National Academy Press). (1994).
[13]-I.F.M., Marai, M.S., Ayyat, U.M., Abd El-Monem, Growth performance and reproductive traits at first parity of New Zealand White female rabbits as affected by heat stress and its alleviation under Egyptian conditions. Tropical Animal Health and Production, 33 (2001) 1–12.DOIhttps://doi.org/10.1023/A:1012772311177
[14]- S.K, Suvarna,, Layton, C., and Bancroft, J.D. Bancroft's Theory and Practice of Histological Techniques, 8th ed. UK: Elsevier Health Sciences. (2019). DOI: https://doi.org/10.1016/C2015-0-00143-5.
[15]-SPSS, 2001. Statistical Software Package for the Social Sciences; SPSS Inc.: Chicago, DE, USA.
[16]- D.B Duncan, Multiple range and multiple F test. Biometrics11:1-42. (1955).
[17]-AOAC, Association of Official Analytical Chemists, Official Methods of Analysis, 21th ed Washington DC USA.(2019).
[18]-S.T., Moschakis, B.S., Murray, C.G., Biliaderis, Modifications in stability and structure of whey protein-coated o/w emulsions by interacting chitosan and gum arabic mixed dispersions. Food Hydrocolloids, 24(1) (2010) 8-17.DOI: 10.1016/j.foodhyd.2009.07.001
[19]-M., Cabuk, M., Yavuz, H.I., Unal, Electrokinetic, electrorheological and viscoelastic properties of Polythiophene-graft-Chitosan copolymer particles Physicochemical and Engineering, Aspects, 510(5) (2016) 231-238 (2016).https://doi.org/10.1016/j.colsurfa.2016.06.014.
[20]-O.P., Sharma, O.M., Bambawale, J.B., Gopali, S., Bhagat, S., Yelshetty, S.K., Singh, R., Anand, O.M., Singh, Field guide Mung bean and Urd bean. Government of India, Department of agricultural and co-operation, NCIPM, ICAR, New Delhi, India (2011).
[21]-D.N., De Assis, V.C., Mosqueira, J.M., Vilela, M.S., Andrade, V.N., Cardoso, Release profiles and morphological characterization by atomic force microscopy and photon correlation spectroscopy of 99m Technetium fluconazole nanocapsules. International Journal of Pharmaceutics, 349 (1-2) (2008) 152–160.doi: 10.1016/j.ijpharm.2007.08.002.
[22]-F.Y.F., Hernandez, S., Khandual, I.G.R., López, Cytotoxic effect of Spirulina platensis extracts on human acute leukemia Kasumi-1 and chronic myelogenous leukemia K-562 cell lines. Asian Pacific Journal of Tropical Biomedicine 7(1) (2017) 14–19. DOI:10.1016/j.apjtb.2016.10.011.
[23]-M.I., Gil, F.A., Tomás-Barberán, B., Hess-Pierce, A.A., Kader, Antioxidant capacities, phenolic compounds, carotenoids, and vitamin C contents of nectarine, peach, and plum cultivars from California. Journal of Agricultural and Food Chemistry, 14; 50 (17) (2002) 4976–4982. doi: 10.1021/jf020136b.

[24]-U.D., Enyidi, Chlorella vulgaris as protein source in the diets of African catfish Clarias gariepinus, Fishes 2(4) (2017). https://doi.org/10.3390/fishes2040017

[25]- M.Y., Hanafi- Bojd, S., Hasheminezhad, E., Alemzadeh, S.M., Derazkola, M., Yousefi, Green synthesis of silver nanoparticles using Trachyspermum ammi fruit extract: Characterization and antibacterial activity, Journal of Nanostructure 13(3) (2023).doi:10.22052/JNS.2023.03.002.

[26]-E.S., Moustafa, W.F., Alsanie, A., Gaber, N.N., Kamel, A.A., Alaqil, A.O., Abbas, Blue-Green Algae (Spirulina platensis) Alleviates the Negative Impact of Heat Stress on Broiler Production Performance and Redox Status. Animals (Basel). Apr 26; 11(5) (2021):1243. https://doi.org/10.3390/ani11051243

[27]-J.J., DiNicolantonio, A.G., Bhat, J., Okeefe, Effects of spirulina on weight loss and blood lipids: a review. Openheart, 7 (1) (2020): e001003.doi: 10.1136/openhrt-2018-001003

[28]- J., Chen, F., Wang, X., Zhou, Y., Cao, Y., Li, C., Li, Bama Miniature Pigs' Liver Possess Great Heat Tolerance through Upregulation of Nrf2-Mediated Antioxidative Enzymes. Journal of Thermal Biology, 67 (2017) 15–21.doi: 10.1016/j.jtherbio.2017.04.012.

[29]- H.K., Kang, H.H., Lee, C.H., Seo, Y., Park, Antimicrobial and Immunomodulatory Properties and Applications of Marine-Derived Proteins and Peptides, Marine Drugs, 17(6) (2019):350.doi: 10.3390/md17060350.

[30]- F.A., Hassan, E.M., Hoballah, M.M., Basyony, S.A., El-Medany, Effect of dietary selenium enriched micro-algae supplementation on growth performance and anti-oxidative status of rabbits under high ambient temperature in summer season. Egypt. Journal of Nutrition and Feeds. 18 (2) (2015) 229–244. DOI:10.21608/ejnf.2015.105111.

[31]- N.D., Sorelle, V., Bertin, T., Hervé, F., Ngoula, Effects of Aqueous Extract of Spirulina platensis on Some Reproductive Performances in Rabbit Does (Oryctolagus cuniculus), Integrative Journal of Veterinary Biosciences, 6(2) (2022) 1–5.DOI: 10.13140/RG.2.2.21864.75526.

[32]- E.B., Rebez, V., Sejian, M.V., Silpa, F.R., Dunshea, Heat Stress and Histopathological Changes of Vital Organs: A Novel Approach to Assess Climate Resilience in Farm Animals. Sustainability 15 (2) (2023), 1242.

[33]-A., Agarwal, S.A., Prabakaran, Mechanism, Measurement, and Prevention of Oxidative Stress in Male Reproductive Physiology. Indian Journal of Experimental Biology, 43(11) (2005), 963–974.

[34]-B.N., Aguanta, A.L., Fuller, M.C., Milfort, S.M., Williams, R., Rekaya, S.E., Aggrey, Histologic Effects of Concurrent Heat Stress and Coccidial Infection on the Lymphoid Tissues of Broiler Chickens. Avian Disease, 62(4) (2018), 345. DOI: 10.1637/11907-052818-Reg.

[35]-R., Deng, T.C., Chow, Hypolipidemic, antioxidant, and anti-inflammatory activities of microalgae Spirulina. Cardiovascular Therapeutics, 28(4) (2010) e33-e43.doi:10.1111/j.1755-5922.2010.00200.x.

[36]-H.T., Shalan, Y.R., Abd El Fattah, The Potential Protective Impact of Spirulina on Cisplatin Induced-Renal Toxicity in Adult Male Albino Rats: Histological, Immunohistochemical and Biochemical Study Egyptian Journal of Hospital Medicine, 85 (2) (2021), 4182-4193.DOI: 10.21608/ejhm.2021.207819.

[37]-A.A., Youssef, A.H., Reda, F.A., Nicola, B., Fulvia, A.A., Rashed, D.A., Adel, H.A., Hani, A.A., Mohamed F.K., Asmaa, E., Wolfgang, Effects of Spirulina platensis and/or Allium sativum on Antioxidant Status, Immune Response, Gut Morphology, and Intestinal Lactobacilli and Coliforms of Heat-Stressed Broiler Chicken, Veterinary Sciences 10(12) (2023), 678. doi: 10.3390/vetsci10120678.

[38]-P., Calella, M., DiDio, G., Cerullo, V., DiOnofrio, F., Gallé, G., Liguori, Antioxidant, immunomodulatory, and anti-inflammatory effects of Spirulina in disease conditions: a systematic review.
International Journal of Food Sciences and Nutrition, 73(8) (2022) 1047-1056.doi: 10.1080/09637486.2022.2137785.

[39]-S.P., Angel, M., Bagath, V., Sejian, G., Krishnan, R., Bhatta, Expression Patterns of Candidate Genes Reflecting the Growth Performance of Goats Subjected to Heat Stress. Molecular Biology Reports 45(6) (2018) 2847–2856. doi: 10.1007/s11033-018-4440-0.

[40]-S., Shaji, V., Sejian, M., Bagath, G.B., Manjunathareddy, E.K., Kurien, G., Varma, R., Bhatta, Summer Season Related Heat and Nutritional Stresses on the Adaptive Capability of Goats Based on Blood Biochemical Response and Hepatic HSP70 Gene Expression. Biological Rhythm Research 48(1) (2017) 65–83.https://doi.org/10.1080/09291016.2016.1232340