JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

IMPACT OF BIOSYNTHESIZED SILVER NANOPARTICLES ON GROWTH, HEMATOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF ZEBRAFISH

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Abstract

The silver nanoparticles were synthesized by Neem *Azadirachta indca* leaves and were characterized by using UV-Vis, SEM, EDAX, XRD, and FTIR. Six different feeds were prepared using different quantiles of silver nanoparticles such as 0(control)2, 4, 6, 8 and 10 µg and common ingredients such as fish meal, groundnut oil cake, wheat flour and tapioca flour. Feed utilization, hematological, and biochemical parameters were estimated after 21 days of feeding trail. The UV-Visible adsorption spectra demonstrates that silver nanoparticles were measured in wavelength with in the 200 – 700nm and exhibits strong adsorption at 227 nm. SEM image was observed at the wavelength of 15.10 nm. EDAX Spectrum recorded two peaks between 2 and 4 KeV. The XRD diffraction peaks are indexed as 77.21, 64.26, 37.86, 31.80 and 27.38.FTIR spectrum of Ag NPs were analyzed in the range of 500 – 4000 cm^{-i} and Silver nanoparticles formation were confirmed 3400, 3027, 1565,998 and 418 cm^{-i}. The condition factor of zebrafish was higher in final when compared to initial. The feed consumption and feed conversion efficiency of zebrafish was higher in feed V and VI The feed conversion ratio of zebrafish was higher in feed IV. Growth and percentage of growth of zebrafish was higher in feed VI.Assimilation and Metabolism of zebrafish was higher in feed V.Haematiological parameters of Zebrafish were decreased form feed I (control) to feed V. Total protein, carbohydrate and lipid in muscle, liver and gill of Zebrafish were higher in feed VI.

Key words: Impact, Silver Nanoparticles, Growth, Hematology, Biochemical, Zebrafish

INTRODUCTION

Nanoparticles has a wide usage potential in aquaculture and sea food industry. It enhancing bioavailability of functional compounds. Currently, the feed additives such as trace minerals in nanoparticles can be used effectively to deal the requirement of minerals in livestock feed. Nanoparticles play a major role in the production of food products against microbial contamination. Nanoparticles are helped as a carrier of nutrients, nutraceuticals, enzymes, food additives and it also enhance the physical, chemical and nutritional quality of feed (Muthuswami Ruby Rajan and Gnanavel Roopashree.,2022). Nanoparticles have wide applications due to its specialized properties that include minute size, high reaction rate, increased surface area and quantum effect. Adding mineral nutrients to the regular fish diet at the nanoscale might so have a tremendous impact not only on growth but also the overall health of the fish. Among nanoparticles, silver nanoparticles are widely used in different fields including medical, food, healthcare, consumer, and industrial purpose due to its unique physical and chemical properties. Silver nanoparticles are mainly used for antimicrobial and anticancer therapy and also applied in the promotion of wound repair and bone healing, growth, anti-diabetic agent and biosectors. Silver nanoparticles exhibit a consistent amount of flexible properties which endorse them for a larger spectrum of applications in biomedicine and related fields. Due to their novel properties, the incorporation of silver nanoparticles into different material like textile and wound dressing and biofilm development.

Silver nanoparticles have received the special interest especially in biomedicine. Ag Nps are famous for its broad-spectrum and highly efficient antimicrobial and anticancer activities. Recently, metal nanoparticles like silver, gold and copper with biological, optical and magnetic interesting properties, within these Ag Nps is the most studied because of it showed antimicrobial potential to bacteria, virus and fungus. Phytogenic feed additives have been incorporated into aqua-feed as natural feed additives to stimulate the fish growth, health, immunity and disease resistance (Harikrishnan Bar *et, al.*, 2009)

Zebrafish is a freshwater fish. Because of its fully sequenced genome, easy genetic manipulation and high fecundity, it is a unique model for biological studies. Zebrafish provides an attractive platform for testing many ingredients to select those with the highest potential of success in aquaculture (Pilar E Ulla *et, al., 2014).* The work related to the biosynthesized silver nanoparticles using neem leaves *Azadirachta indica* on growth and

biochemical characteristics of zebrafish is totally wanting. Hence the present study was carried out.

MATERIALS AND METHODS

MATERIALS

Neem *(Azadirachta indca)* leaves, Silver nitrate (AgNO3), Sodium hydraoxide (NaOH) were used for the synthesis of silver nanoparticle. The leaves were collected from the campus of The Gandhigram Rural Institute – Deemed to be University, Gandhigram, Dindigul, Tamil Nadu, India. The chemicals used for the synthesis of AgNps were purchased from the Loba chemicals, India. The required glass wares were washed and rinsed with distilled water, dried and sterilized in hot air oven.

PREPARATION OF LEAF EXTRACT

The aqueous leaf extract of *A. indica* were used for the green synthesis of AgNps. It involves several steps. For this extract preparation, first 10 grams of the neem leaves were collected and washed thoroughly with tap water and then distilled water. After that the leaves were dried and cut into small pieces and crushed with mortor and pestle. These substances were boiled with 100ml of distilled water for 20 minutes in a magnetic stirrer in temperature between 60 and 80°C. After 20 minutes of boiling in the conical flask containing the boiled substance and cooled at room temperature. Then filtered with Whatman No.1 filter paper twice. Here, the purified aqueous leaf extract was obtained.

BIOSYNTHESIS OF SILVER NANOPARTICLES

1.69 g of AgNO3 was dissolved separately in 100 ml of distilled water. 10 ml of *A. indica'*s aqueous leaf extract were added with 90 ml of AgNps solution. This is heated with hot plate at 80˚C for 3hours with constant stirring. pH of these solution should be neutral (7-9). NaOH was added (5 to 10ml) for fixation of pH. The formation of the AgNps was determined by the change in colour from dark brown to black. After that the green synthesized nanoparticles were allowed to settle down at room temperature. Then centrifuged these nanoparticles at 15,000 rpm for 10 minutes. This process were repeated thrice by using distilled water and ethanol. Finaly the AgNps pellets were obtained. These pellets were further dried at room temperature, and the powdered using mortor and pestle then AgNps were gained.

CHARACTERIZATION OF SILVER NANOPARTICLES

(a)UV- Visible spectroscopy

UV-Visible analysis of silver nanoparticles absorbance were in peak ranging between 220 - 700 nm.

(b)Scanning Electron Microscope (SEM)

Scanning electron microscope projects and scans a focused stream of electrons over a surface to create an image. The electrons in the beam interact with the sample, thereby producing various signals that can be used to obtain information about the surface's topography and composition. The sample of AgNps morphology were observed by using the scanning electron microscope.

(c)Energy Dispersive X-ray Spectroscopy (EDAX)

It is an analytical technique used for the elemental analysis or chemical characterization of a sample through a microscope.

(d)X-ray Diffraction (XRD

XRD was used to determine the phase and crystalline structure of biosynthesized silver nanoparticles. These analysis was done by x-ray diffractometer using CuKa radiation with the wavelength of 1.540Å at 40 kv.

(e)Fourier Transforms Infrared Spectroscopy (FTIR)

The spectra of the silver nanoparticles were obtained by Fourier transform infrared spectroscopy in the diffuse reflectance mode at a resolution of 4 with kBr pellets.

COLLECTION AND ACCLIMATION OF ZEBRAFISH

For this study, the zebrafish (0.101±0.02g) were collected from Adithya pets, Palani road, Dindigul, Tamil Nadu, India and transported to the laboratory in polythene bags filled with oxygenated water. These fishes were acclimated in plastic trough for 10 days at room temperature. During this period, fishes were fed with trainee feed, that contains fish meal, groundnut oil cake, wheat flour and rice brain in the form of dry pellets.

SELECTION OF FEED INGREDIENTS AND EXPRIMENTAL FEED PREPARATION

The raw materials for feed preparation were selected based on its ability to supply nutrients. After knowing the protein content by Micro- Kjeldhal method the feed were prepared. The protein sources are fish meal and groundnut oil cake, carbohydrate sources are wheat flour and tapioca flour, vegetable oil was used as binding agents. Supplevite - mix was also added. The components that used for feed preparation were dried, powdered and sieved through 425 - micron sieve. The ingredients were weighed and mixed thoroughly with 200 – 240 ml of distilled water. These mixture were put into the autoclave for 15 minutes at 100˚C and cooled. Then fish oil, sunflower oil, supplevite mix, sodium chloride, sodium benzoate and various quantity of AgNps (2,4,6,8,10 µg) were mixed with feed, then it was extruded using pelletizer. These pellets were dried in room temperature. After that, the dried formulated feed were kept in air tight container at - 20˚C until used to prevent contamination(Table 1).

Table -1: Composition of different components in the experimental feed (g/ 50gm) of zebrafish

Ingredients	Experimental Feeds					
	Feed I (control)	Feed II	Feed III	Feed IV	Feed V	Feed VI
Fish meal	16.87	16.87	16.87	16.87	16.87	16.87
Groundnut Oil Cake	16.87	16.87	16.87	16.87	16.87	16.87
Wheat flour	5.625	5.625	5.625	5.625	5.625	5.625
Tapioca	5.625	5.625	5.625	5.625	5.625	5.625
Fish oil	1	1	1	1	1	1
Sunflower oil	1	1	1	1	1	1
Supplevite- mix	0.5	0.5	0.5	0.5	0.5	0.5
Sodium Chloride	0.5	0.5	0.5	0.5	0.5	0.5
Sodium Benzoate	0.5	0.5	0.5	0.5	0.5	0.5
Silver Nanoparticles	0	2 µg	4 µg	6 µg	8 µg	10 µg

EXPERIMENTAL DESIGN FOR GROWTH STUDIES

For the present study uniform size of Dania rerio (0.101± 0.02 g) were selected. These selected fishes were introduced in the glass tank having a capacity of 15 liters. Five fish were introduced in each tank. For each treatment triplicates were maintained. During this study, the fishes were fed on ad libitum diet of the prepared feed twice a day for 1 hour each from 8.30 – 9.30 and 3.30 – 4.30 pm. The unfed were collected after one hour of feeding without disturbing the fishes. The unfed was dried to constant weight. The fecal matter was collected daily before changing the water with least disturbance to the fishes and dried at 95˚

C. Approximately 70% of water in the tank were replaced with tap water. The experiment was continued for 21 days. On the 21st day length and weight of the fishes were measured in live condition. The fishes were sacrificed for taking muscle, gill and liver from all experiments takes for further analysis.

FEED UTILIZATION AND GROWTH PARAMETERS

Feed utilization and Growth parameters such as condition factor, feed consumption, feed conversion efficiency, feed conversion ratio, growth, percentage growth, assimilation, metabolism, gross and net growth efficiency were estimated after 21 days.

HAEMATOLOGICAL PARAMETERS

After 21 days, blood samples were collected from cordial vein in right side of the fish. Complete blood parameters such as WBC, Polymorph Neutrophils, Lymphocytes, Eosinophiles, Haemoglobin, RBC, Haematocrit (PCV), Platelet count, Blood protein, MCV, MCH were estimated.

BIOCHEMICAL CHARACTERISTICS

Biochemical characteristics such as total protein (Lowry et al. 1951), carbohydrate (Carrol et al. 1956), and lipid (Folch et al., 1957) were estimated in liver, muscle and gill of Zebrafish.

RESULTS AND DISCUSSION

The UV-Visible absorption spectroscopy is widely used technique to examine the optical properties of the nanosized particle. The absorbance spectra of Silver nanoparticles were measured in wavelength within the range 200 to 700 nm. It exhibits the strong absorption band at 227 nm as shown in figure 1. Anal K Jha and Prasad, (2010) reported that the absorption spectra of the sample were measured by a computer - controlled UV - Visible Spectrophotometer. The silver surface plasmon resonance was observed at 449 nm. Scanning electron microscopy shows that nanoparticles formed of clusters because of the adhesive nature of spherical shaped appearance as shown in figure 2. It can be seen that Ag particles from both sources are in the form of agglomerates. Geoprincy *et al.*, (2013) reported that size and morphology of silver nanoparticles were analyzed using SEM. The presence of Silver were revealed in synthesized nanoparticles by EDAX spectral analysis. EDAX spectrum recorded on the silver nanoparticles is shown at two peaks located between 2 KeV and 4 KeV and another peak of C element was located on the between 0.1 and

0.2(Fig.3). Nehead Ahmad and Seema Sharma., (2012) reported that EDAX analysis was carried out to determine the elemental composition and stereochemistry of biosynthesized silver nanoparticles. Silver nano crystallites display on optical absorption band peak at approximately 3 KeV. he XRD technique is used for structure and phase analysis of all compounds. The XRD diffraction peaks of silver nanoparticles are indexed as 77.21, 64.26, 37.86, 31.80 and 27.38 which is represented in Figure 4. The clear and sharp diffraction peaks confirmed that the prepared compounds are pure with high degree of crystallinity. Mehta, *et al.*, (2017) reported that data was taken for the 2^{θ} range of 30 to 80 degrees with a step of 0.0202 degree. Four peaks at ɵ values of 38.2901, 44.5583, 64.8185 and 77.4383. The FTIR spectrum of silver nanoparticles was analyzed in the range of 500- 4000cm-1. The FT-IR measurement was carried out for identifying the functional groups of bioactive components based on the peak value in the region infrared radiation. Silver formation was confirmed 3400, 3027, 1565, 998 and 418 cm-1 bands have alcohol, alkele, diketone, alcohol and alkyl halides compound(Figure 5). Mallikarjuna *et al.*, (2011) reported that FTIR measurements were carried out to identify the biomolecules of capping. The spectrum was recorded in the range of 4000 – 400 cm^{-i}. the functional groups and bands are observed at 3419, 2923, 1618, 1376,1163, 1113 and 1059 cm^{-i}.

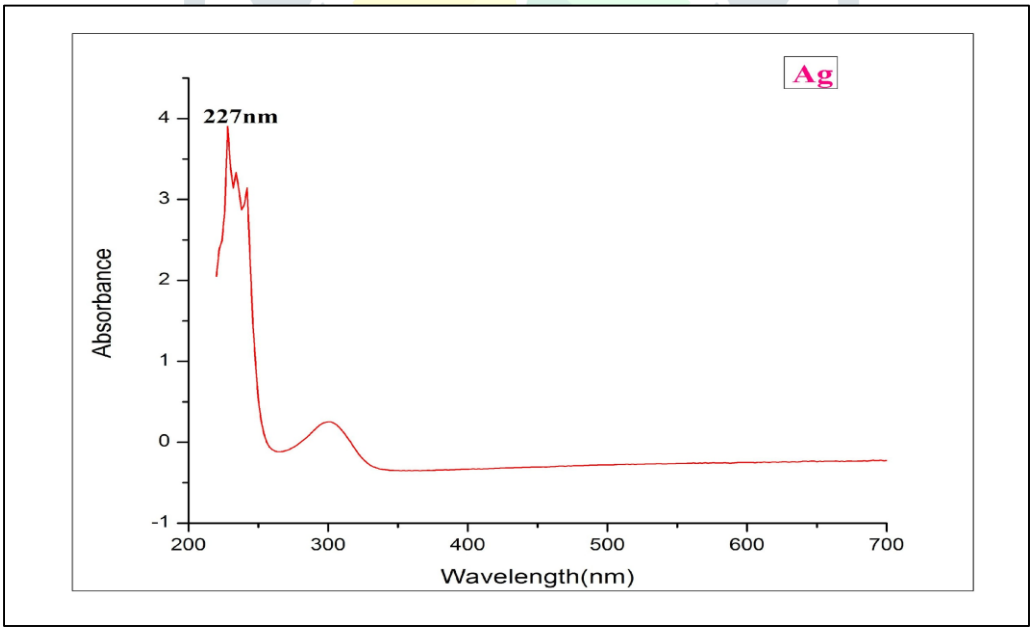


Figure 1. UV-Visible Spectroscopy Analysis of Silver nanoparticles

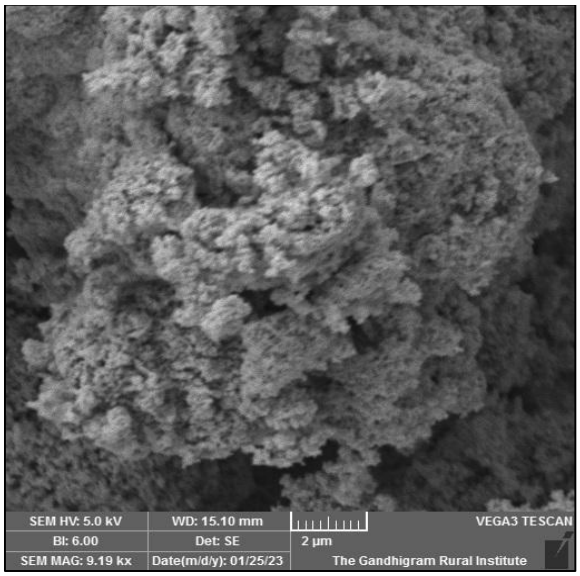


Figure 2. SEM Analysis of Silver nanoparticles

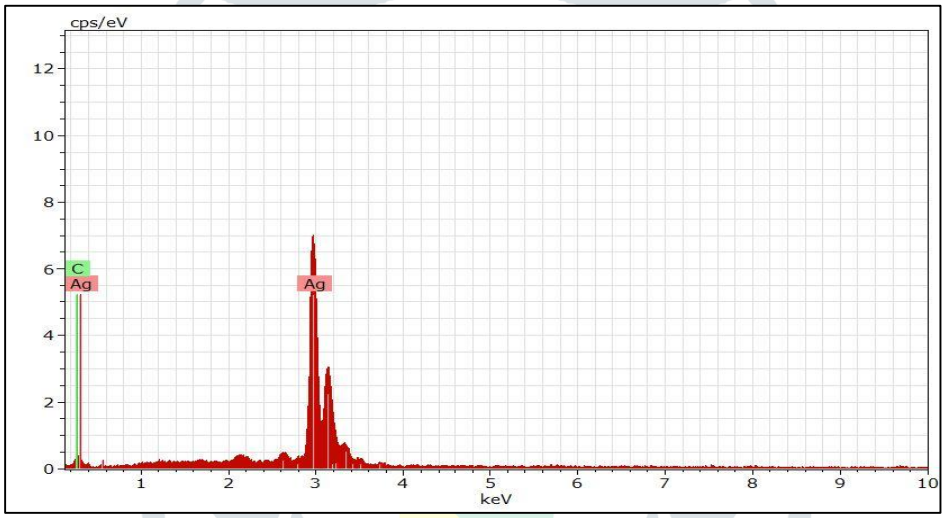


Figure 3: EDAX Image of Silver nanoparticles

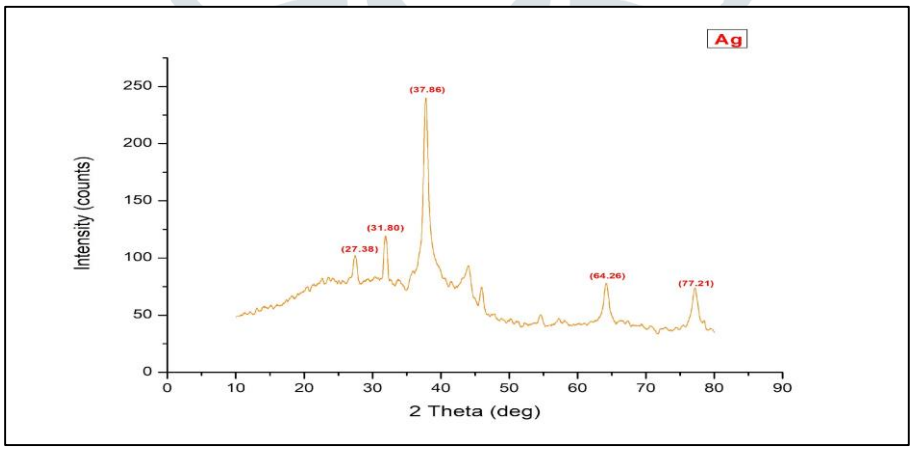


Figure 4. XRD Image of Silver nanoparticles

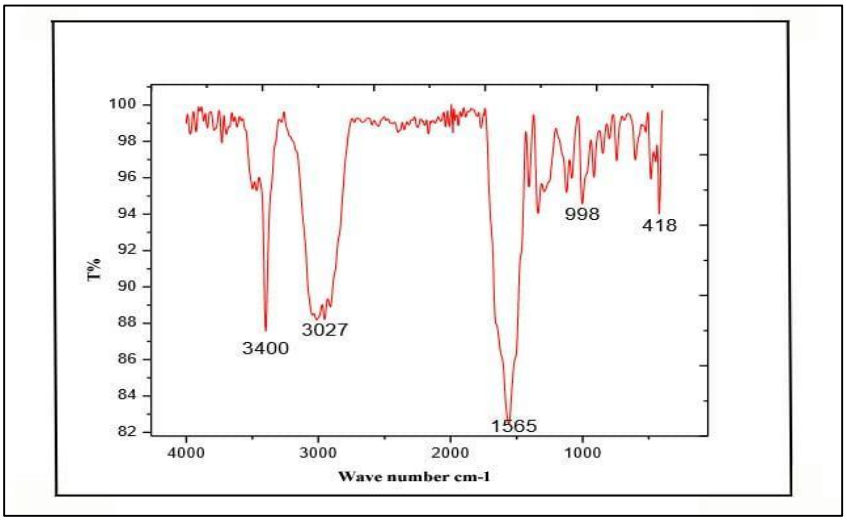


Figure 5. FTIR Image of Silver nanoparticles

The condition factor of Zebrafish reared in different feeds were presented indicated in Tables 2. The final condition factor is increased in all feeds. Srinivas *et al.*, (2016) reported an increase in condition factor of *Macrobrachium rosenbergii* post – larval feed with 40 / kg $^{-1}$ of iron oxide nanoparticles in the feed. The different feed utilization and growth parameters are presented in table 3. The ANOVA (Analysis of Variance) of growth parameters (Feed consumption, Growth, Gross Growth Efficiency, Net Growth Efficiency) are presented in is given (Table 4). The feed consumption of Zebrafish was higher in feed V containing 8µg of AgNPs in the feed. Muthuswami Ruby Rajan and Raja Rohini (2021) reported that the feed consumption of Mrigal was higher in feed IV containing 15 mg / g^{-1} of ZnO NPs. The feed conversion efficiency of Zebrafish were higher in feed VI containing 10 µg of silver nanoparticles. Growth and percentage of growth of Zebrafish was higher in feed VI containing 10 µg of silver nanoparticles in the feed. Thirunavukkarasu Muralisankar *et al.*, (2016) reported that the growth of *Macrobrachium rosenbergii* was higher in copper supplemented feed. The assimilation and metabolism of Zebrafish was higher in feed V respectively. The gross growth efficiency and net growth efficiency were higher in feed V containing 8 µg of silver nanoparticles. Muthuswami Ruby Rajan and Saravanakumar Sakthiprabha (2022) reported that the assimilation, metabolism, gross growth efficiency and net growth efficiency were higher in feed V containing iron oxide nanoparticles.

Table 2. Condition factor of Zebrafish

Feeds	Initial	Final
EX.Feed (Control)	1.176 ± 0.304	1.453 ± 0.478
EX. Feed II	0.973 ± 0.204	1.516 ± 0.474
EX. Feed III	1.051± 0.553	1.36 ± 0.206
EX. Feed IV	0.986 ± 0.196	1.38 ± 0.065
EX. Feed V	1.13 ± 0.252	1.273 ± 0.106
EX. Feed VI	1.12 ± 0.060	1.236 ± 0.011

Table 3. Feed utilization and Growth parameters of Zebrafish in relation to different quantity of Silver nanpoparticles. Each value is the average (± SD) performance five individuals in triplicates reared for 21 days

Parameters	Experimental Feeds					
	Feed I (Control)	Feed II (2ug)	Feed III (4ug)	Feed IV (6ug)	Feed V (8ug)	Feed VI (10ug)
Feed consumption (g/g live wt /21days)	1.33±0.1	1.5±0.2	2.83±0.89	2.96±0.46	3.26±0.5	3.1±0.92
Feed Conversion Efficiency	0.05±0.02	0.04±0.05	0.08±0.04	0.12±0.02	0.1±0.01	0.18±0.01
Feed Conversion Ratio	3.35±0.53	4.05±0.32	6.21±0.45	7.05±0.52	3.85±0.71	2.15±0.45
Growth	0.25±0.05	0.36±0.08	0.48±0.04	0.68±0.05	0.58±0.05	0.76±0.03
Percentage Growth	1.52±0.12	2.02±0.16	0.53±0.21	0.9±0.10	1.8±0.23	3.2±0.31
Assimilation (g/g live wt /21days)	1.2±0.16	1.53±0.61	1.45±0.34	0.87±0.25	1.6±0.25	1.2±0.38
Metabolism (g/g live wt /21 days)	1.6±0.20	1.2±0.3	1.18±0.52	1.3±0.12	2.3±0.24	1.53±0.48
Gross Growth Efficiency (%)	24.2±0.75	27.2±1.8	29.6±2.6	30.4±3.5	44.03±1.7	26.1±2.7
Net Growth Efficiency (%)	19.3±2.32	26.3±2.67	24±1.44	18.5±2.54	28.6±1.73	20.51±1.7

Table 4. ANOVA (Analysis of Variance) of Growth parameters (Feed consumption, growth, gross growth efficiency, net growth efficiency) of Zebrafish

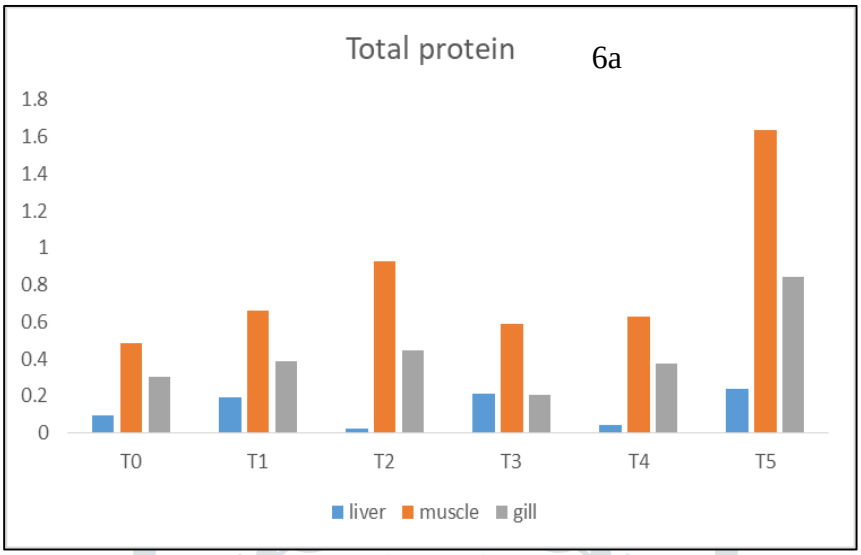
Parameters	Source of variation	Sum of Squares	DF	Mean Squares	F	SIG
Feed consumption	Between Groups Within groups Total	1.16256 1.35743 3.34055	03 10 15	0.24321 0.05008	5.68	0.0121 S
Growth	Between Groups Within groups Total	0.8065 0.88665 2.1165	04 11 17	0.10314 0.12237	8.98	0.00087 S
Gross Growth Efficiency	Between Groups Within groups Total	1329.78 24354.87 5213.52	03 10 12	448.78 50.765	7.55	0.0016 S
Net Growth Efficiency	Between Groups Within groups Total	1652.44 1228.8 3846.06	03 16 13	325.98 33.897	8.78	0.0007 S

The hematological parameters of Zebrafish is presented in Table 5. Haematological parameters are very helpful in judgment of health condition of fish species. The RBC and WBC count was higher in feed IV. Haemoglobin content was higher in feed IV. Haematocrit is higher in feed IV and lower in feed II. The platelets are decreased in increasing quantities of silver nanoparticles respectively. Zadmajid and Mohmmadi (2017) reported that the control fish exposed to Ag NPs showed significantly lower to RBC count, haematocrit and haemoglobin content. Mohsen Imani *et al.*,(2015) reported that silver nanoparticles shows decreased in RBCs, Hbs, Hct, WBCs and MCH.

Table 5. Haematological parameters of Zebrafish

Blood parameters	Feed I	Feed II	Feed III	Feed IV	Feed V	Feed VI
WBC (cells /cumm)	10,400	8,100	9,200	18,000	3,900	2,800
Polymorph Neutrophils (%)	0	26	0	22	0	19
Lymphocytes (%)	0	58	0	66	0	67
Eosinophiles (%)	0	16	0	12	0	14
Haemoglobin (gm/dl)	0.4	0.3	0.2	1.0	0.2	0.1
RBC count (million/cumm)	0.1	0.2	0.1	0.4	0.1	0.1
Haematocrit (PCV) (%)	08	0	0.5	08	03	0.2
MCV	0	0	0	0	0	0
MCH	0	0	0	0	0	0
MCHC	0	0	0	0	0	0
Platelets count (Lakhs/cumm)	74,000	44,000	48,000	60,000	34,000	16,000

Biochemical parameters such as total protein, carbohydrate and lipid content (mg/g) in muscle, gill and liver of Zebrafish is higher in feed VI containing 10 µg of silver nanoparticles(Figure 6).Ashouri *et al.,* (2015) reported that the selenium nanoparticles in the feed increase the protein, carbohydrate and lipid content of muscle, gill and liver of *Oreochromis mossambicus.*



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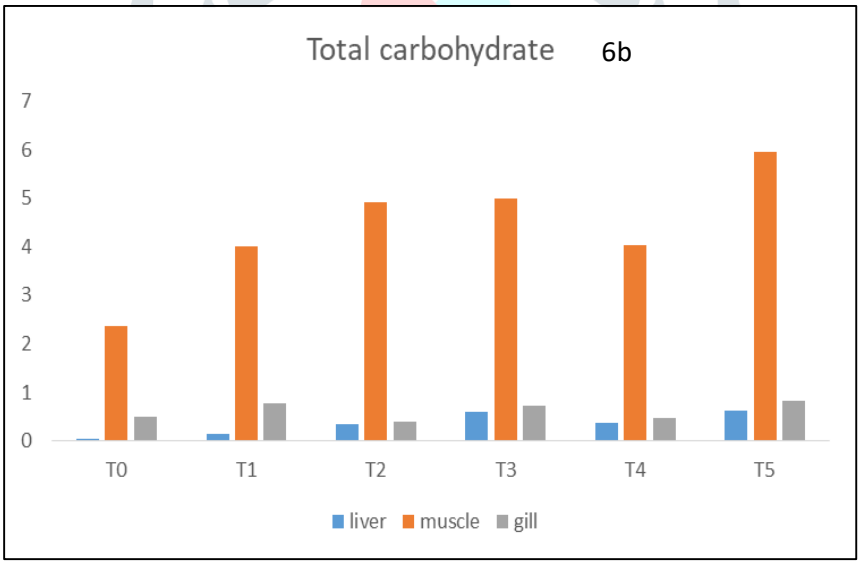

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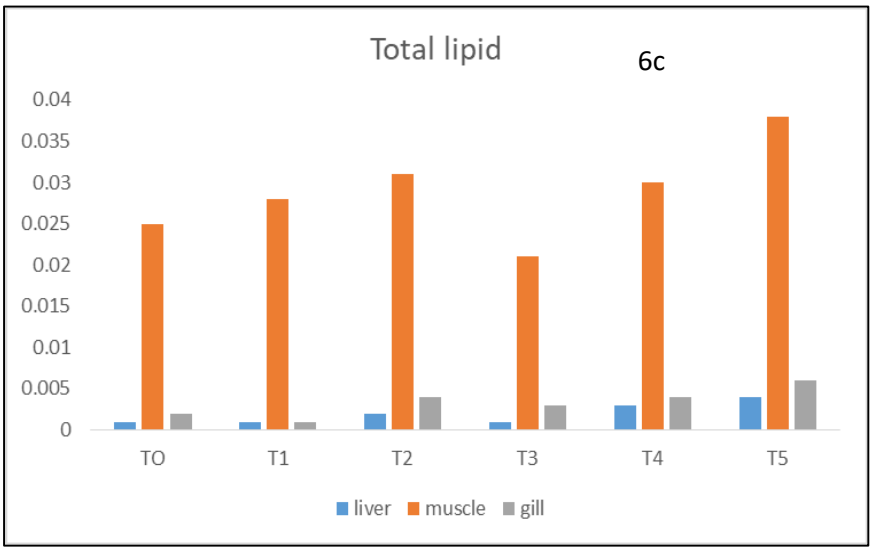

Figure 6 . Total Protein(6a), Carbohydrate(6b), and lipid(6c) in liver, muscle and gill of Zebrafish

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