BIOSYNTHESIZED SILVER NANOPARTICLES INCORPORATED FEED ON GROWTH AND HAEMATOLOGICAL CHARACTERISTICS OF ZEBRA FISH DANIO RERIO

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Abstract

Silver nanoparticles were biosynthesized using Indian Pennywort Centella asiatica and characterized using UV-Visible Spectroscopy, SEM, EDAX, FT-IR, and XRD. Different quantity of biosynthesized Silver nanoparticles such as 1, 2, 3, 4, and 5 mg were prepared by using a fish meal, groundnut oil cake (GNOC), wheat flour and tapioca flour. Feed utilization parameters of Zebrafish such as Feed Consumption, Feed Conversion Ratio, Feed Conversion Efficiency, Growth, Percentage Growth, Assimilation, Metabolism, Gross Growth Efficiency and Net Growth Efficiency were estimated after 21days.After experimental period haematological parameters of zebrafish such as total RBC, Hb, Hct, total WBC and total Platelets were estimated. The UV-visible absorption spectra demonstrate that Ag NPs were measured in wavelengths within 400 to 500 nm and exhibited strong adsorption at 296 nm and 425 nm. SEM image showed that the Silver nanoparticles were observed at the wavelength range from 6.81nm(scalebar: 10µm). EDAX spectrum showed two peaks located between 0.2 KeV and 3.0 KeV. FT-IR spectrum was observed at the wavelength range from 4000 to 900cm^{-1}. XRD image shows that the diffraction peaks were indexed as 28.09, 32.62,38.28, 46.60, 55, 57.90, 64 and 69. The final condition factor of Zebrafish was decreased compared to the initial condition factor. The feed consumption and feed conversion efficiency of Zebrafish was higher in feed V. The feed conversion ratio was best in feed V. Growth and percentage growth, assimilation and metabolism of Zebra fish was higher in feed V. Gross and Net growth efficiency were higher in feed III. All the haematological parameters decreased with increased quantities of Silver nanoparticles.

Keywords: Silver nanoparticles, *Centella asiatica,* growth, haematology, Zebrafish

Introduction

In recent decades various environmental challenges have been mitigated due to a boom in nanotechnologies and nanomaterials. Many adverse effects have been associated with chemical synthesis methods due to the presence of some toxic chemical absorbed on the surface. Eco friendly alternatives to chemical and physical methods are biological ways of nanoparticles synthesis using microorganisms, enzymes, fungus, and plants or plant extracts. Among nanoparticles, silver nanoparticles have several applications such as biomedicine, drug delivery, ointments, food industry, cream, agriculture, textile industry and in water treatment (Bo Rao *et al.,* 2017). *Centella asiatica* is one of the important traditional plants to the family Apiacea. It is commonly known as Indian pennywort, Asiatic Pennywort, Spade leaf, Coin wort (or) Gotu Kola and used to treat various disorder, minor wounds and are found in tropical, and subtropical countries like India, Srilanka and Bangladesh (Arpita Roy and Bharadvaja, 2017). In Ayurvedha, *Centella asiatica* is considered as a " medhya rasayana" i.e agent used for memory improvement, the ability to revitalize nerves and brain cells, anxiety treat mental fatigue and eczema(Brinkhaus *et, al.,*2000).The leaves, roots, stems of *Centella asiatica* contains the bioactive compounds such as alkaloids , saponins, flavonoids ,tannins, sterols and phenolic compounds and act as a defense mechanism to protect the plants from various diseases. The *Centella asiatica* has phytochemical activity that is responsible for the reduction of silver ions and capping of AgNPs (Jayappa *et .al.,* 2020). The herb is possessing anti-tumor, anti-inflammatory, anti-oxidant, anti-depressant, anti-diabetic, anti-microbial, neuroprotective, cardioprotective and wound healing properties (Gallego et.al., 2014). It is extensively used in pharmaceuticals, food and cosmetic industries. Aquaculture is a rapidly growing global food industry that plays a vital role in meeting the demand for animal protein. However, the industry is hampered by disease, chemical contamination, environmental degradation, and ineffective feed utilization. To address these challenges, new technologies are being developed, including nanotechnology. Nanotechnology has shown great potential for improving aquaculture through the use of novel nano tools. Zebrafish (*Danio rerio*) is an important vertebrate and model organism. It is widely used in the assessment of chemicals, nanotoxicology studies due to its small size, ease of maintenance, rapid development and available genomic information (Spence *et al.,* 2008). It is a small robust fish and can breed all around a year, so it can be grown easily. Zebrafish has increasing importance in biomedical research particularly in the model for human disease and for the screening of therapeutic drugs, also it is easily available, inexpensive and can be maintained properly. (Kiruba Daniel *et al.,* 2011). Good nutrition is essential for cost-effective and high-quality production of healthy animals in animal production systems, particularly in fish farming where feed accounts for approximately 50% of variable production costs. Recent advancements in fish nutrition have led to the development of new, balanced commercial diets that promote optimal fish growth and health, including species-specific formulations that support the growing demand for affordable, safe, and high-quality fish and seafood

products. The nutrients required by fish for growth, reproduction, and other normal physiological functions are similar to those of land animals. They need protein, minerals, vitamins growth factors, and energy sources. Growth is a characteristic feature of living beings. It is an important aspect of the biology andlife history of fish, and quantification of growth is frequently a crucial part of fisheries research and management (Weatherley and Gill,1987). Biochemical tool is useful for monitoring health status, detecting illness and responses to therapy. Haematological studies furnish an index of physiological changes in fish and the fishblood acts an impressive tool for detection of alterations in the tested organism (Adhikari *et al.,* 2007). The work related to biosynthesis of Silver nanoparticles using Penny Wort *Centella asiatica* and their incorporation in the feed on growth, and haematological characteristics of Zebra fish is totally wanting. Hence the present study was carried out.

Methodology

For the experimental study, Silver nanoparticle was biosynthesized by using the leaf extract of Indian pennywort (*Centella Asiatic*a) as a reducing agent. Fresh leaves were collected from Perundurai,Erode. The surface of the leaves was cleaned with running water to remove debris and other contaminated organic contents and dried at room temperature The leaves from *Centella asiatica* were pulverized into a fine powder using a mechanical grinder and stored in an airtight container for further analysis. The powdered material was loaded into themain chamber of the Soxhlet extractor by packing inside the thimble. The distillation flask was filled with the extraction solvent. The distillation flask was attached to the heating mantle and the Soxhlet extractor was connected to the round bottom flask and after that, a condenser was placed to condense the evaporated solvent which was kept cool for effective condensation. This setup temperature was adjusted to below the respective solvent boiling point. Some desired compoundsdissolved as the solvent warmed, and when the extraction chamber was filled with the solvent, thesiphon emptied the chamber and the return the solvent to the round bottom flask. This cycle was repeated for about 5-6 hours till the solvent of the chamber turned colourless assuming that most of the compounds were extracted. After extraction solvent was evaporated and the extract was concentrated using a rotary vacuum evaporator. Qualitative analysis was performed for *Centella asiatica* leaf extract.

Preparation of leaf extract

Using 10 g of fresh leaves of *C. asiatica* were weighed and transferred into 250mL beakers containing 100mL distilled water and boiled by microwave for about 30 min, sieving it and storing it for the synthesis of AgNPs.

Synthesis of Silver nanoparticles

Silver nitrate (AgNO3) was collected from the Department of biology, The Gandhigram Rural Institute (Deemed to be a university), Tamil Nadu, India. The reaction mixture was prepared by adding 10 ml of the callus extract to 90 ml aqueous solution of 1 mM AgNO3 (9:1 ratio-optimized concentration) in 250 ml Erlenmeyer flask and incubated in a dark place at 350 C for about 48h. The primary detection of reduction of silver ions (Ag) to AgNPs (Ag) was carried out by observing the colour change of the reaction mixture from light yellow to dark brown. Confirmation of the synthesis and characterization was carried out by spectrophotometric measurements.

Characterization of Silver nanoparticles

The synthesized nanoparticles were characterized by UV-Vis Spectrophotometer (JASCO-V-530), Scanning Electron Microscope (SEM)(LEO 1455 VP), Energy Dispersive X-ray detection instrument (EDAX)(HORIBA 8121-H), Fourier Transform Infrared Spectroscopy JASCO(FTIR-6200) and X-ray Diffraction (XRD) (SHIMADZU Model XRD ¬ 6000).

Collection and acclimation of Danio rerio

For growth studies Zebrafish fingerlings (1.340±0.054g) were collected from Aqua Garden Fish Farm, Kadachanenthal, Madurai, Tamil Nadu, India (Plate 1) and transported to the laboratoryin polythene bags filled with oxygenated water. Fishes were acclimated in glass aquaria (60x45x45cm) for a period of 15 days at 28±2^{0}C. During acclimation, a feed containing fish meal, groundnutoil cake, wheat flour and rice bran were used in the form of dry pellets.

Selection of feed ingredients and experimental feed preparation

The raw materials are selected based on their ability to supply nutrients to the fish. The major raw materials used in this study are Fish meal and ground nut oil cake, tapioca powder, wheat flour. After knowing the protein content by Micro-Kjeldhal method, the feed was prepared in (Table 1). The ingredients were weighed and mixed thoroughly with 130-150 ml of distilled water. The mixed feed stuff was put in an autoclave for 30 min at 100^{0}C and cooled. After cooling, fish oil, sunflower oil, supplevite-mix mix, sodium chloride, sodium benzoate and different quantity of silver nanoparticles were mixed with the feed and it was extruded with the help of a pelletizer. The formulated feed was kept in an air tight container at -20^{0}C until used to prevent contamination (Table 2).

Table 1: Protein level of major ingredients

S.No	Ingredients	Protein
1	Fishmeal	58
2	Groundnut oil	44
3	Wheat flour	11
4	Tapioca	03

Table 2: Composition of different ingredients in the experimental feed(g\100gm) of Zebra fish

Ingredients	Feed I (Control)	Feed II	Feed III	Feed IV	Feed V	Feed IV
Fish meal	33.75	33.75	33.75	33.75	33.75	33.75
GNOC*	33.75	33.75	33.75	33.75	33.75	33.75
Wheat flour	11.25	11.25	11.25	11.25	11.25	11.25
Tapioca	11.25	11.25	11.25	11.25	11.25	11.25
Fish oil	2	2	2	2	2	2
Sunflower oil	2	2	2	2	2	2
Supplevite-mix	1	1	1	1	1	1
Sodium chloride	1	1	1	1	1	1
Sodium benzoate	1	1	1	1	1	1
Ag-Nanoparticles	0	1mg	2mg	3mg	4mg	5mg

Haematological Parameters

Blood samples were collected from fishes after 21 days, from cordinal vein in right side ofthe fish using the disposable insulin syringe fitted with the fine needle, without harming the fish.The syringe and needle were moistened with EDTA. The collected blood was then transferred into an Eppendorf tubes containing 0.1 N EDTA. Complete blood parameters such as RBC, WBC, Platelet count, Hemoglobin (Hb), Haematocrit (Hct), were estimated after 21 days.

Results and Discussion

The UV visible absorption spectra is a technique used to characterize the silver nanoparticles. The absorbance spectra of AgNPs were measured in wavelength within the range from 400 nm to 500nm. The sharp bands observed close to 296 and 425 nm. (Figure 1).Dixit and Khan *et al.,*(2017) reported the sharp absorption peak at 425 nm from the UV-Vis analysis of AgNPs synthesized by biological method. Scanning electron microscopy shows that the nanoparticles formed the clustered form because of the adhesive nature of spherical shaped appearance as shown in Figure 2. Arpita Roy *et al.,(*2017) reported that the SEM images indicates the spherical like shape of synthesized nanoparticles. EDAX Spectrum recorded on the AgNPs is shown as three peaks located between 0.2 Kev and 3 Kev (Figure 3). Those maxima are directly related to the silver characterized line K. The maximum peak located on the spectrum at 3 KeV clearly comes from Silver. The maximum peak located on the spectrum at 0.2 KeV clearly comes from oxygen. The FTIR spectrum of silver nanoparticles was analyzed in the range of 4000-900 cm^{-1} The FT-IR analysis was carried out for identifying the functional groups of active components based on the peak value in the region of infrared radiation. The functional group of silver nanoparticles was confirmed with bands having 3299, 1750,1589, 1394, 1044, and 818, were associated with N-H Band. Carboxylic acid, C=C Stretching, O-H Bending, C=N Stretching, C=C Bending are respectively. (Figure 4). The peaks at 3451, 1592, 1384 is due to O-H stretching, symmetric and anti-symmetric stretching of COO-respectively(Sreelekha et al., 2021). The XRD technique is used for structure and phase analysis of all compounds. The XRD diffraction peaks of silver nanoparticles are indexed as 28.09, 32.62, 38.28 ,46.60, 55, 57.90, 64 and 69 which is represented in Figure 5. The clear and sharp diffraction peaks confirmed that the prepared compounds are pure with high degree of crystallinity. Shani Raj *et al., (*2018) subjected the phytochemically synthesized AgNPs to XRD analysis and reported that all the peaks are very sharp and indicates crystalline nature of product and no extra peak is present in XRD pattern which shows that synthesized product does not contain any impurities. Bothe Thokchom *et al.,(*2023) characterized the green synthesised AgNPs from *Centella asiatica* leaf extract using XRD. The analyzed materials are finely ground and homogenized and the average bulk composition is determined (Khan et al.,2018).

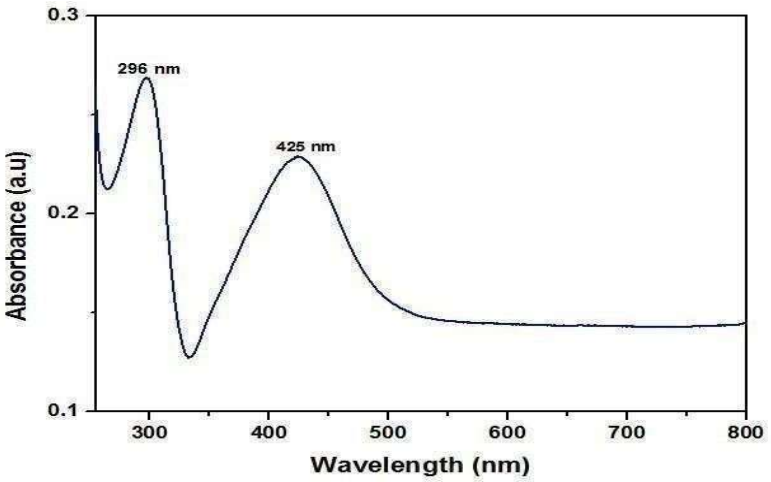

Figure 1. UV-Vis Image of Silver nanoparticles

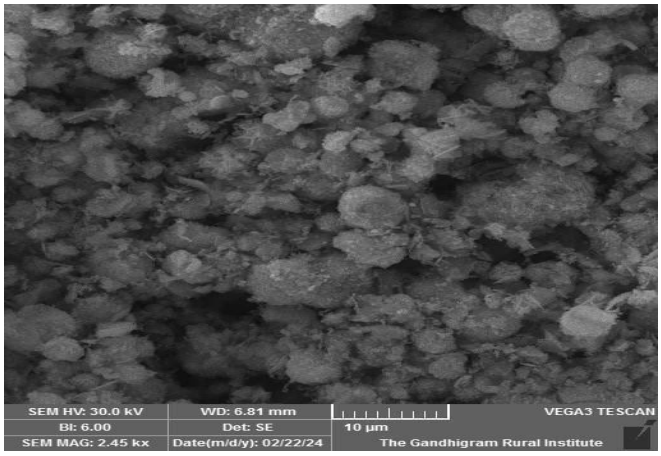

Figure 2. SEM Image of Silver nanoparticles

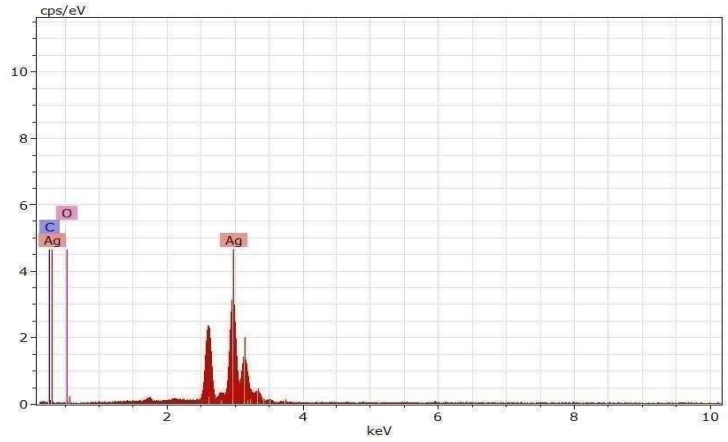

Figure 3: EDAX Spectrum of Silver nanoparticles

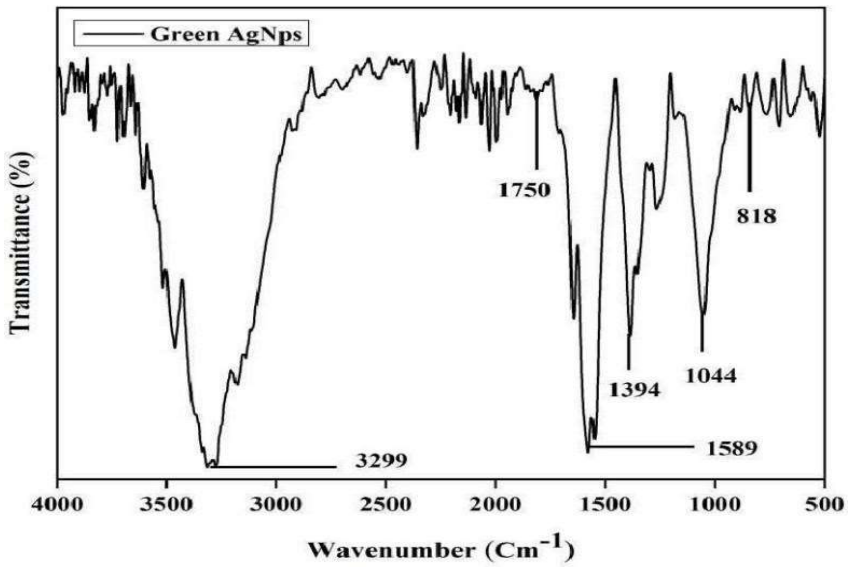

Figure 4. FTIR Spectrum of Silver nanoparticles

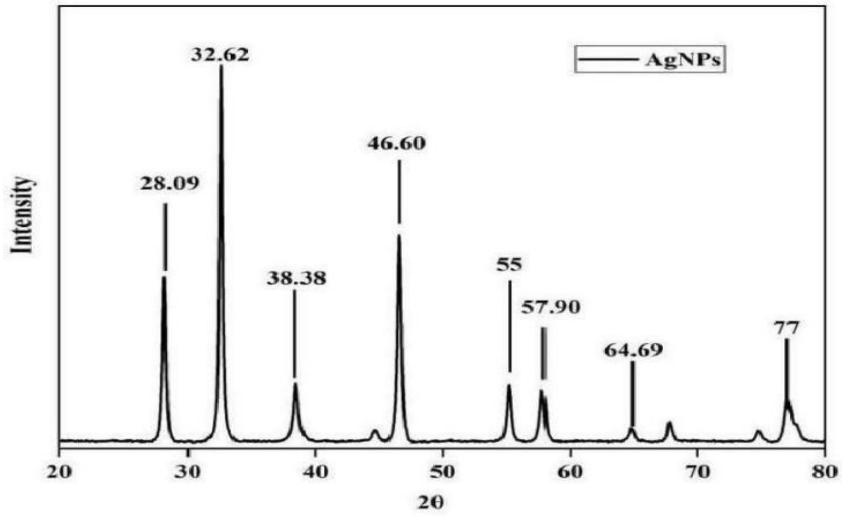

Figure 5. XRD Spectrum of Silver nanoparticles

The Condition factor of Zebrafish reared in different feeds were presented in Table 3. The condition factor (K) of zebrafish was estimated for comparative purposes to assess the feed. The final condition factor of zebrafish was increased in all the feeds supplemented with Silver nanoparticles. Srinivasan *et al*., (2016) reported an increase in the condition factor of *Macrobachiium rosenbergii* post-larvae fed with 40g / kg^{-1} of iron oxide nanoparticles in the feed. A Similar condition factor was also reported in koi carp fed with zinc oxide nanoparticles (Soundariya and Rajan 2021). The different feed utilization and growth parameters are presented in Table 4. ANOVA (Analysis of Variance) of growth parameters (Feed

consumption, Gross Growth Efficiency, Net Growth Efficiency) were presented in Table 5. Feed consumption of zebrafish was higher in feed V containing 4 mg of AgNPs in the feed. The feed consumption of Zebra fish increased when the concentration of Silver nanoparticles increased in the feed. Rajan and Rohini (2021) reported that the feed consumption of Mrigal was higher in feed IV containing 15 mg/g^{-1} of zinc oxide nanoparticles. The feed conversion efficiency of zebrafish was higher in feed V. Growth and the percentage growth of zebrafish were higher in feed V . Muralisankar *et al*., (2016) reported that the growth of *Macrobrachium rosenbergii* was higher in copper supplemented feed. Fasil et al., (2021) reported that the selenium and zinc oxide supplementation enhanced growth performance in zebra fish. The assimilation and metabolism of zebrafish were higher in feed V and feed VI respectively. Sangeetha and Rajan (2021) reported that the assimilation of Koi carp was higher in feed IV containing 30 mg of iron oxide nanoparticles in the feed. Rajan and Meenakumari (2023) reported that the assimilation and metabolism of common carp were higher in feed containing 40 mg of magnesium oxide nanoparticles. The gross growth efficiency of zebrafish was higher in feed VI containing 5mg of AgNPs in the feed. The net growth efficiency was higher in feed V. Rajan and Rohini (2021) reported that the gross growth efficiency was higher in *Cirrhinus mrigala* fed with feed IV containing 15mg of ZnO NPs in the feed. Rajan and Meenakumari (2023) reported that the gross and net growth efficiency of Common carp was higher in feed V containing 40 mg of magnesium oxide nanoparticles.

Table 3. Feed utilization and Growth parameters of Zebrafish in relation tothe different quantities of silver nanoparticles. (Each value is the average (± SD) performance of five individuals in triplicates reared for 21 days)

Parameters	Experimental Feeds					
	Feed I (controll)	Feed II (1mg)	Feed III (2 mg)	Feed IV (3 mg)	Feed V (4 mg)	Feed VI (5 mg)
Feed consumption (g/g live wt./21 days)	0.45±0.19	0.52±0.09	0.32±0.10	0.66±0.19	0.68±0.07	0.58±0.09
Feed conversion efficiency	0.39±0.17	0.45±0.34	0.75±0.50	0.82±0.30	1.07±0.77	0.68±0.30
Feed conversion ratio	5.32±1.49	6.22±2.94	4.60±3.08	7.24±3.90	9.29±4.31	3.44±0.38
Growth	0.05±0.01	0.06±0.02	0.16±0.12	0.1±0.03	0.17±0.04	0.13±0.04
Percentage growth	9.80±4.33	9.86±0.98	19.22±12.21	17.23±7.32	26.74±1.68	21.72±5.93
Assimilation(g/g live Wt./21 days)	2.90±1.06	3.05±0.97	2.86±1.80	3.19±0.91	3.34±0.41	3.27±0.61
Metabolism (g/g live wt./21 days)	2.85±1.06	2.99±0.99	2.70±1.72	3.09±0.92	3.21±0.38	3.10±0.57
Gross growth efficiency (%)	13.03±7.54	19.64±11.64	32.91±21.19	16.33±7.17	19.75±5.35	29.29±3.51
Net growth efficiency (%)	1.93±0.93	2.25±1.65	7.06±4.79	3.35±1.47	4.04±4.01	4.30±1.77

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

Parameter	Source	SS	DF	MS	F	Significance
Feed Consumption	Between groups Within groups Total	.268 .225 .492	5 12 17	0.054 0.019	2.8 59	0.063 **NS**
Growth	Between groups Within groups Total	.035 .005 .040	5 12 17	0.0O7 0.001	16.69	0.005 **S**
Gross Growth Efficiency	Between groups Within groups Total	428.028 132.125 560.153	5 12 17	85.606 11.010	7.775	0.005 **S**
Net Growth Efficiency	Between groups Within groups Total	31.529 10.879 42.408	5 12 17	6.306	6.955	0.005 **S**

Haematological parameters are very helpful in the judgment of the health condition of fish species. The WBC count of zebrafish is gradually decreased as the quantity of AgNPs increased from the feed I to feed VI. The platelets are decreased with the increasing quantity of silver nanoparticles in the feed(Table 5). Shah and Altindag (2005) reported that the haematological parameters such as haematocrit, Hb, RBC and WBC are used to assess the functional status of the oxygen-carrying capacity of the bloodstream and have been used as indicators of metal pollution in the aquatic environment. In the present study, the haematological analysis such as RBC and Haemoglobin of Zebra fish exposed to bulk silver nanoparticles was significantly decreased. Abdel –Tawwab *et al*., (2007) reported the increase of blood parameters with a high concentration of selenium nanoparticles supplemented in the feed of African catfish, *Clarias gariepinus.* Anand Sadanandan Ramya *et al*., (2015) reported that the haematological parameters were gradually increased with different doses of iron oxide nanoparticles fed on Indian major carp. Faiz et al ., (2015) reported that haematological characteristics of grass carp fed with ZnO supplemented diet showed a significant decrease in WBCs, Hb, HCT, MCV, MCH values but an increase in RBC and MCHC values.

Table 5. Haematological parameters of Zebrafish

Blood parameters	Feed I	Feed II	Feed III	Feed IV	Feed V	Feed VI
RBC count (millions/cumm)	0.34	0.3	0.26	0.21	0.15	0.11
Haemoglobin (gm/dl)	1.01	0.9	0.8	0.7	0.5	0.4
Haematocrit (%)	2.81	2.6	2.1	1.86	1.50	1.16
WBC count (Cells/cumm)	16100	14000	9800	8100	6900	800
Platelets count (Lakhs/cumm)	1.28	92000	72000	65000	60000	51000

Conclusion

The present study concluded that the Feed V containing 4mg of biosynthesized Silver nanoparticles is suitable for the growth of Zebrafish.

Conflict of Interest

There was no conflict of Interest

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