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Research Article

Dietary *Chlorella vulgaris*, silver nanoparticles, and their mixture modulate Nile tilapia (*Oreochromis niloticus*) growth, profitability, antioxidant status, bioenergetics, amino acid profile, and palliate physical stresses

Matter A.F.[1*], Soror E.I.[1*], Shehab A.[2], Farid O.A.[3], Abd-El Hamed A.M.[4], Abo-Gamil Z.H.[5], Khalid A.[6], Soliman T.S.[7], Youssuf H.A.[1]

1Aquatic Animal Medicine Department, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh, Qalyubia, Egypt
2 Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh, Qalyubia, Egypt
3 Department of Physiology, National Organization for Drug Control and Research, Giza, Egypt
4 Economic and Farm Management, Department of Animal Wealth Development, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh, Qalyubia, Egypt
5 Animal and Poultry Production, Department of Animal Wealth Development, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh, Qalyubia, Egypt
6 Department of Basic Engineering Sciences, Faculty of Engineering (Shoubra), Benha University, Cairo, Egypt.
7 Institute of Natural Science and Mathematics, Ural Federal University, Ekaterinburg 620000, Russian Federation
*Co-correspondence: emansoror@yahoo.com; ayamatter29@yahoo.com

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Abstract

This study investigated the effect of silver nanoparticles (AgNPs) and Chlorella vulgaris nanoparticles (ChVNPs) on growth, oxidant stress, bioenergetics, muscle amino acids, and profitability in Nile Tilapia (Oreochromis niloticus) under hypoxia and cold stress. We divided 180 fingerlings (10.03± 0.5 g) into four equal groups with three replicates (15 fish /replicate). Nile tilapia received basal diet (control), basal diet with ChVNPs (5g/kg diet), basal diet with AgNPs (0.02 mg/kg diet), or basal diet with both ChVNPs and AgNPs (mixed group). Each group was subdivided into three subgroups with stress exposure (hypoxia or cold) or without stress (control). After 45 days, growth performance and feed utilization of fish exposed to AgNPs showed significant improvement. The mixed treatment significantly (p<0.05) reduced the stress-induced elevation of total cholesterol (TC), triglycerides (TG), and cortisol. Hypoxia and cold stresses induced oxidant stress marked by significant reduction (p<0.05) in SOD, CAT, and GSH levels and significantly increased in MDA, NO, and GSSG. Basal diet with both ChVNPs and AgNPs (mixed group) significantly altered the oxidant-antioxidant status. The hypoxia and cold stress modulated the muscle amino acid profile by reducing ISO, LEU, METH, and TAU and increasing LYS. In basal diet with both ChVNPs and AgNPs (mixed group), muscle ARG, HIS, LYS, PHEN, VAL and ASP were significantly decreased (p< 0.05) in compared with other treated groups, whereas; ISO, LEU, METH, TUA, SER, GLY, ALA, and PRO were significantly increased (p<0.05) in compared with other treated groups. Stress significantly decreased (p<0.05) muscle ATP and increased ADP and AMP that were modified by the basal diet with both ChVNPs and AgNPs (mixed group). The fish diet supplemented with AgNPs achieved the highest economic profits compared to other treatments. However, basal diet with both ChVNPs and AgNPs (mixed group) is recommended to alleviate adverse stressors effect.

Introduction

Aquaculture is one of the rapidly growing food industries worldwide, which is crucial in reducing the rising demand for animal protein (Okeke *et al*., 2022). However, the aquaculture sector is struggling with several production-related issues, including disease outbreaks, expensive feed, physical stressors, and water pollution (García-Beltran *et al*., 2020). As a result, aquaculture must utilize modern technologies to tackle these problems. Rapidly expanding and important economic sector, nanotechnology enables new products with innovative and special functions. The efficacy of using nanoparticles (NPs) is attributed to their higher surface area, which increases their action. Due to their exceptional qualities, NPs have found extensive usage in a variety of industries, including agriculture, medicine, and water treatment (Mabrouk *et al*., 2021). Interestingly, it has been discovered that nanoparticles improved aquafeeds by boosting the proportion of fish feed components that pass across the gut tissue and into the fish, as opposed to going through the fish digestive system unaffected (Onuegbu Chris *et al*., 2018). Moreover, NPs have been shown to be successful at delivering micronutrients, increasing the amount of feed produced in a given length of time, and promoting growth (Khosravi-Katuli *et al*., 2017). Recently, nanomaterials have developed into useful technologies for diagnostics and therapy in human medicine and veterinary medicine because they are identical in size to biomolecules within living cells and viruses (Sanvicens and Marco, 2008). Silver nanoparticles have been studied as anti-infection remedies, and food additives (Barakat and Yousry, 2016) through direct application to experimental animals (Das *et al*., 2020) and can be applied to shrimp to control infections caused by microbes (Camacho-Jiménez *et al*., 2020). AGNPs possess low toxicity to human cells, high thermal stability and low volatility (Durán *et al*., 2007).

The green microalgae, *Chlorella vulgaris* (ChV), is a member of the Chlorellaceae family and Order Chlorellales. (Al dayel *et al*., 2020). In both freshwater and marine ecosystems, it is widely dispersed (Hodač *et al*., 2016). *Chlorella* species have tiny, spherical, or ellipsoidal cells with cup-shaped chloroplasts that are positioned parentally. It is thought that this alga is a good source of phytohormones (Tarakhovskaya *et al*., 2007) and essential nutrients. The protein of this alga contains indispensable amino acids, rich in beta-carotene and chlorophyll (Krienitz *et al*., 2015). They are also rich in vitamin A, thiamin (B1), riboflavin (B2), Pyridoxine (B6), niacin (B3), and cyanocobalamin (B12), L ascoebic acid, minerals such as magnesium, potassium, phosphorus, copper, zinc, iodine, cobalt, calcium, and iron (Sawant and Mane, 2018). Therefore, *Chlorella* had potential impact on growth performance, immuno-modulation, antioxidant defense mechanism, and tissue rebuilding (Guzman *et al*., 2003). In addition, *Chlorella* extract demonstrated antimicrobial properties (Acurio *et al*., 2018). The primary constituent of *Chlorella vulgaris* is cellulose, which also includes additional groups such as phenolic hydroxyl and benzene rings. These functional groups all

have single electron pair configurations. These functional groups were disseminated on the emulsion's surface when nano-scale micro emulsion was generated in the aqueous phase, demonstrating a potent ability for adsorption (Al dayel *et al*., 2020). Annamalai and Nallamuthu (2016) used *C. vulgaris* aqueous extract in the green synthesis of silver nanoparticles and demonstrated its inhibitory impact on several harmful microorganisms. The use of ChV as a nanoparticle is a new approach that was proposed on the basis of the concept of efficient properties of nanoparticles in absorption and nutrient delivery.

Nile Tilapia has high marketability, quick growth, and adequate food conversion. Additionally, it has a good tolerance for bad water quality, is a great fish for intensive culture, and is resistant to several diseases. Nile tilapia is the main Egyptian aquaculture species contributing for more than 65% of fish production (Elsheshtawy *et al*., 2019). However, Tilapia production faces several constraints, particularly water quality and physical stresses in the era of climate change. Poor water quality decreased fish production, increased costs and increases the risk of disease outbreaks (Soliman and Yacout, 2016). Therefore, the use of feed additives to mitigate the deleterious effect of different physical stresses is crucial to improve the productivity and meat quality of Nile Tilapia, which is the most important source of human protein for Egypt. The combined effect of both ChVNPs and AgNPs as feed additives in physically stressed fish aquaculture is not yet investigated in Egypt. Therefore, this study aimed to investigate the combined effect of dietary supplementary of AgNPs and ChVNPs (alone and cmbined) on the oxidative stress, serum biomarkers, energy metabolism, and amino acid profile of fish muscle of *Oreochromis niloticus* exposed to hypoxia and cold stresses. Additionally, growth performance and profitability were determined after feeding AgNPs and ChVNPs each alone and in combination.

Materials and methods

Preparation and characterizations of silver nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) have been generated by chemical reduction method as reported by Iravani *et al.* (2014). The size and shape of AgNPs was measured by TEM JEOL JEM-2100 high resolution transmission electron microscope at an accelerating voltage of 200 kV, respectively (Abdelmawgoud *et al*., 2022). The phase purity and crystalline structure of AgNPs were explored using X-ray diffraction (El-Desouky *et al*., 2022). The dose of the AgNPs (0.2 mg/kg diet) used in this study was selected based on previous studies (Jha *et al*., 2022)

Preparation of ChVNPs

In the Egyptian Nanotechnology Center (EGNC), *Chlorella vulgaris* powder (SHANA Company, Benha, Egypt) was used green synthesis of ChVNPs using the ball mill method as previously described (Elabd *et al*., 2020).

Fish and experimental diet design

This study was *approved* by Animal Care and Use Committee, Faculty of Veterinary

Medicine, Benha University under ethical approval number BUFVTM 07-01-23.

A total number of 180 apparently healthy 55 days age Nile tilapia, *Oreochromis niloticus* fingerlings (10.32±1.1 g weight and 6. 1± 0.5 cm length) were obtained from private fish farms in El Abbassa- Sharkia Governorates and transported alive to wet laboratory of aquatic animal Medicine Department, Faculty of Veterinary Medicine, Benha University. The fish were kept in well prepared glass tanks (750 L) supplied with decholorinated tap water and pumped using air pump (Sicalls, Pietes, Italy). Random samples were scarified using MS222 (100 mg/L using sodium bicarbonate as a buffering solution) and examined mycologically and bacteriology to insure healthy condition. Then, the remaining fish were classified into four groups and left one week for acclimation prior to the experiment. During the experimental study, fish were fed on pelleted commercial fish diet containing 30% crude protein. The diet was provided daily as 6% of fish body weight. Water parameters were adjusted as follows; water temperature at 28±2°C; dissolved oxygen at 6±0.5mg/L; ammonia concentration at 0.53±0.07 mg/L and pH at 7±0.2. The water was renewed at a rate of 25% every 24 hours. Fish feces and feed wastes were removed everyday by siphoning (Shaw *et al*., 2022).

According to the National Research Council (NRC, 1993), the diets were prepared to fulfill the nutritional requirements of Nile tilapia. Table 1 shows the composition of the basal diet used in the feeding trial. The fish diets were made by carefully mixing all the ingredients for 15 minutes and then adding water and oil to produce a doughy, moist mass. The dough mixture was then pelleted without the use of steam to create 2 mm diameter sinking pellets. Finally, the pellets were dried at room temperature using the techniques described in (He *et al*., 2016) and kept until use in sterile, clean plastic bags at –20°C. The fish fed three times a day for 6 weeks at 6% of their body weight (9:00 am, 1:00 pm and 5:00 pm). The uneaten feed was collected 2 hours after eating to accurately estimate the feed intake.

Table 1: Ingredients and composition of the experimental basal diet.

Ingredients	**%**
Yellow corn	20
Soybean meal (44% protein)	24
Corn gluten (60% protein)	4.4
Fish meal	18
Rice bran	12.8
Wheat bran	14
Wheat flour	2.15
Soybean oil	2
Molasses	2
Choline chloride	0.05
Vitamin and mineral premix**	0.35
Vitamin C	0.025
Nutrient specification	%
Crude protein	30.1
Crude lipids	6
Crude fiber	5.21
Calcium	1.41
Total phosphorus	0.84
Lysine	1.73
Methionine	0.63
Threonine	1.15
Cystine+Methionine	1.09
Arginine	1.91
Gross energy, kcal kg^{-1} diet	4180

** Premix provided each kg of feed with Biotin=0.025 mg; Folic Acid = 1 mg; Niacin = 20 mg; Pantothenic acid = 8 mg; Vitamin A = 7000 IU; Vitamin B1 = 1 mg; Vitamin B12 = 0.01 mg; Vitamin B2 = 4 mg; Vitamin B6 = 1 mg; Vitamin D = 1400 IU; Vitamin E = 10 mg; Vitamin K3 = 3 mg; Cobalt = 0.01 mg; Copper = 10 mg; Iodine = 0.05 mg; Iron = 15 mg; Manganese = 40 mg; Selenium = 0.01 mg; Zinc = 40 mg.

The substances used in the experiment as feed additives were AgNPs powder (99.5% purity - MKN-Ag- 090) where the AgNPs size was declared by the manufacturer to be uniformly of diameters <100 nm and ChVNPs. Fish were randomized into four dietary treatment groups (45 fish/group) with 3 replicates (15 fish/replicate). The first group received only a basal diet (control) with no additives. The 2nd group was fed on a basal diet with ChVNPs (5 g/kg diet) as previously recommended (Abdel-Tawwab *et al*., 2022). The 3rd group fed on a basal diet with AgNPs (0.02 mg/kg diet) and the 4th group (mixed group) fed on a basal diet with ChVNPs (5 g/kg diet) and AgNPs (0.02 mg/kg diet) as additives.

Determination of growth performance parameters and expected weight

After counting the fish and a period of fasting for 24 h, we determined both the initial weight and length (at the start of experiment) and the final weight and length (at the end of experiment) after being dried using filter papers. We used the obtained data to calculate the weight gain (WG), the specific growth rate (SGR), the length gain and the feed conversion ratio (FCR) as previously described (Elabd *et al*., 2020). In addition, we calculated the expected weight of tilapia after 20 weeks in the experimental groups according to Dampin *et al*. (2012).

Stressors challenge

To induce cold stress, the fish were transferred from a water temperature of 28±2°C to 12°C pre-cooled aerated water in the 750-L tanks for 30 min. To induce hypoxia, the aeration was shut off until the dissolved oxygen was around 1.5–3.0 mg/L and the signs of hypoxia were monitored as fish surfacing, gasping, accumulating at the water inlet and rapid movement of operculum (Elabd *et al*., 2020)

Blood sample collection and biochemical analysis

The blood samples were collected using heparinized syringes from fish following euthanasia by using 250 mg/l in water tricaine methane sulfonate (MS222) as an anesthetic solution (Syndel Laboratories, British Columbia) as previously described (Soaudy *et al*., 2021). Plasma was separated by centrifugation of the blood samples at 2062 xg at 4°C for 5 min. Plasma was used for the determination of TC, TG, AST, ALT, and cortisol by using commercial kits from Diamond Diagnostics Company, Egypt.

Determination of musculature amino acids profile

Samples of musculature were prepared, homogenized, centrifuged, and purified as previously described (Salah *et al*., 2019) and the filtrate portion was derivatized. Amino acid standards (Sigma-Aldrich, St. Louis, MO, USA) and derivatized meat samples were placed into HPLC (Agilent HP 1200 series apparatus, Santa Clara, CA, USA) constructed with Nova-PakTM C18 column (4 - m, 3.9 - 4.6 mm) for separation and measurement of free amino acids as mentioned earlier (Ali and Elgoly, 2013).

Estimation of oxidative biomarkers, and bioenergetics in the liver

Liver samples were collected, weighed, processed, and homogenized according to Refaey *et al*. (2023). The adenosine

triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were measured in the liver in accordance with the method described by Abd-Elrazek and Ahmed-Farid (2018). In this work, the amounts of malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG), and nitric oxide in the liver tissue were measured using HPLC (Agilent HP 1200 Series Apparatus, USA) (Faizan *et al*., 2014). The spectrophotometric method was used to measure the activity of superoxide dismutase (SOD) and catalase (Aebi, 1984).

Determination of the cost and return parameters

The cost and return parameters were determined for 100 fish per dollar. We used the equations approved by Phiri and Yuan (2018) to determine different cost parameters including total fixed cost (TFC), total variable cost (TVC), and total cost (TC), and the return parameters including total returns (TR) and net profit (NP).

Statistical analysis

The two-way analysis of variance (ANOVA) using SPSS 16.0 software was applied to determine the stress and treatment effect. The growth and profitability parameters were analyzed by one-way ANOVA among dietary groups. We used the Tukey HSD module to test the significance between different dietary groups. The means were considered significantly different when $p<0.05$.

Results

The properties of AgNPs and ChVNPs

The AgNPs appeared brown in color; optical absorption spectra were about λ max ~412 nm The TEM images of the AgNPs with different scales appeared spherical in shape with the average size of 10±2 nm (Fig. 1).

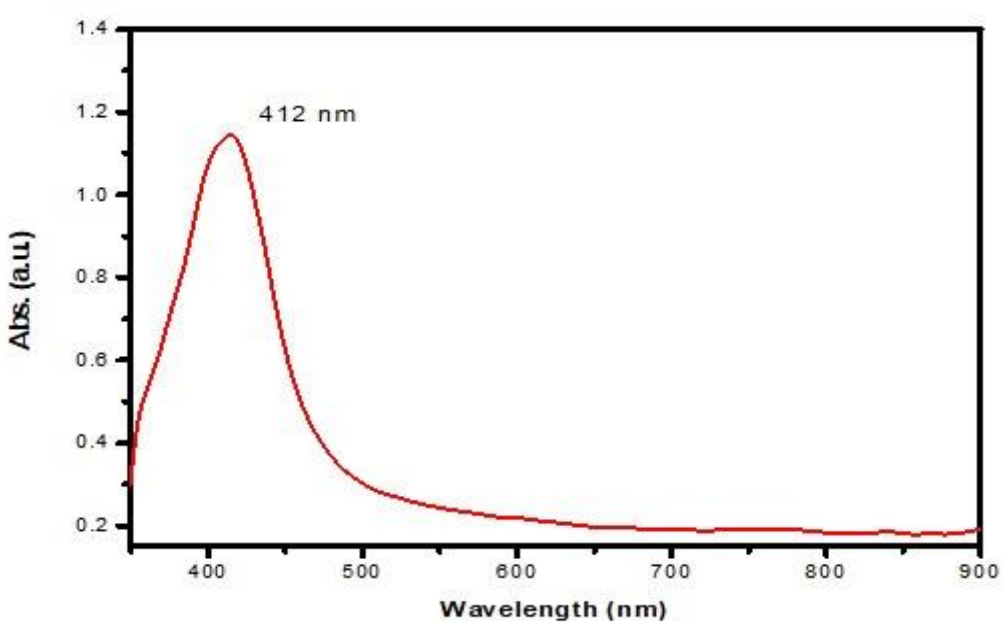

Figure 1: UV-Vis absorption spectra obtained on an Ocean Optics USB2000+VIS-NIR Fiber optics spectrophotometer showing the spectrum of AgNPs (λmax ~412 nm).

Additionally, the chosen area of the electron diffraction patterns indicates the existence of the single-crystalline character of the formed AgNPs (Fig. 2). X ray diffraction (XRD) was used to analyze the structural characteristics of the synthesized AgNPs.

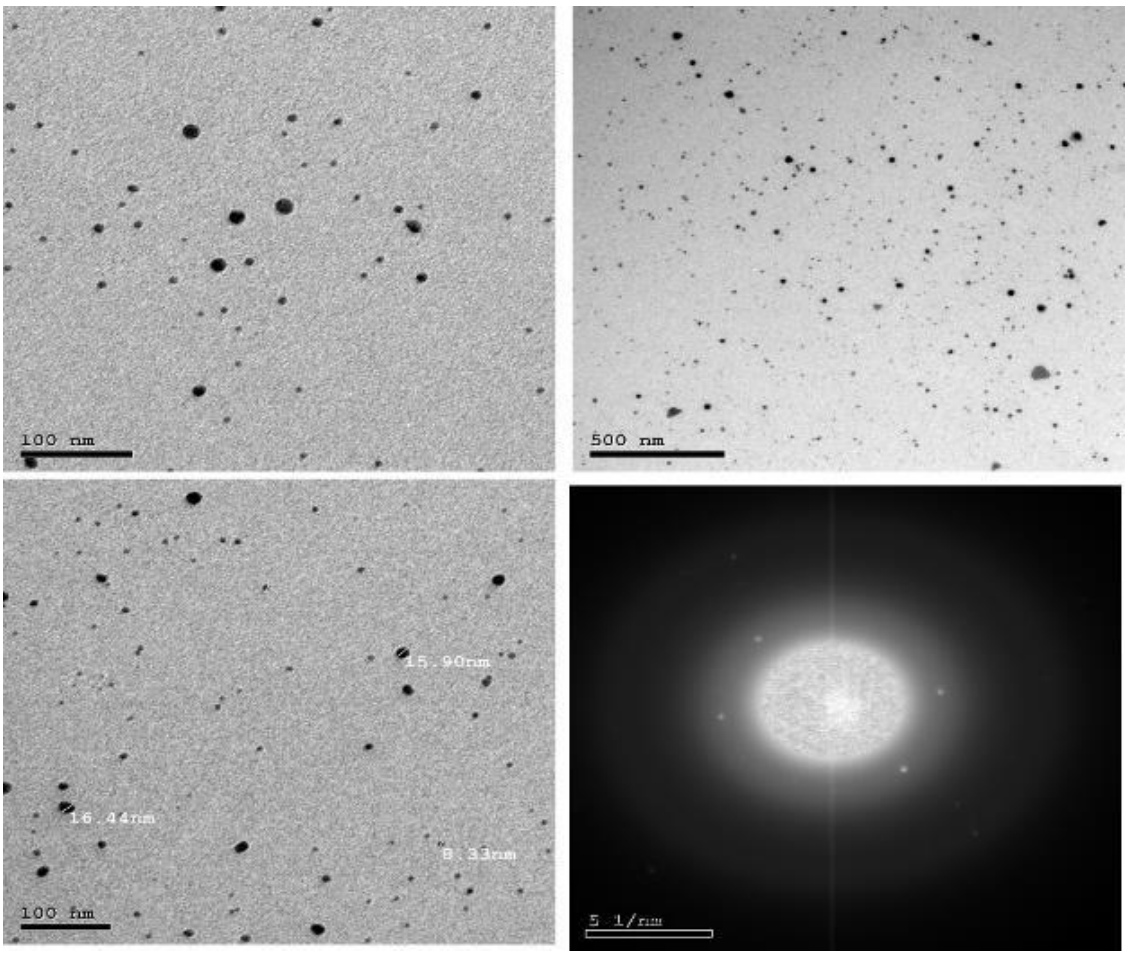

Figure 2: The Size and Shape of AgNPs determined by TEM that were performed on JEOL JEM-2100 high resolution transmission electron microscope at an accelerating voltage of 200 kV, respectively. The TEM image of AgNPs at different scales and SAED pattern appeared spherical with an average size of 10±2 nm.

The XRD patterns, as illustrated in Figure 3, revealed five distinct peaks at 38.07°, 44.27°, 64.55°, 77.56°, and 81.43° correspond to the (111), (200), (220), (311), and (222) planes respectively of the AgNPs with reference code (JCPDS-XRD Cards no: 1100136 QM). The particle size (Z) of the supported AgNPs was calculated using Debye–Scherrer's formula [$Z= 0.89\ \lambda/Y \cos(\theta)$], where λ is the X-ray wavelength, Y is the full-width at half-maximum and θ is the scattering angle. The calculated particle size Z, from the XRD humps for the as-synthesized AgNPs sample was about 9 nm (Fig. 3). The dislocation density (U) $=1/Z^2$ of AgNPs is about 0.012 nm^{-2}. The measured average particle size of AgNPs is roughly in the same range as the matching TEM and XRD data. The Data of AgNPs is

shown in Table 2. The properties of ChVNPs are presented in Figures 4 and 5.

Changes in growth performance indicators

The data presented in Table 3 revealed that the final body weight, body weight gain, weight gain rate and SGR in fish supplemented with nanoparticles either alone or mixed was significantly increased (p<0.01) compared to the control group.

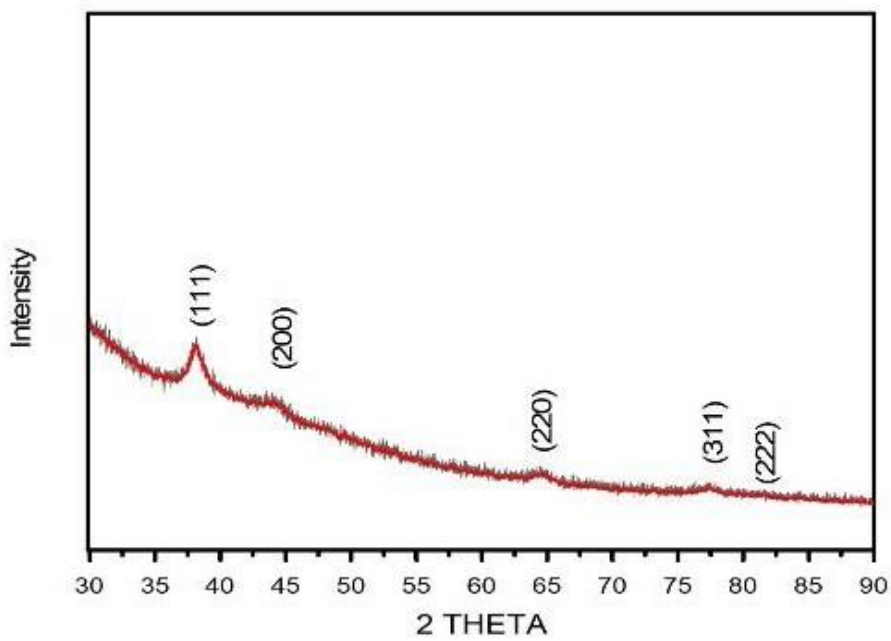

Figure 3: The phase purity, crystalline structure and d-spacing of the as-prepared AgNPs using X-ray diffraction.

Table 2: XRD data of AgNPs.

2θ^0	**d, Å spacing**
38.07	2.36
44.27	2.04
64.55	1.44
77.56	1.22
81.43	1.18

The XRD patterns of the synthesized AgNPs show five distinct peaks**.**

The feed conversion ratio (FCR) was significantly reduced in fish fed on diets supplemented with AgNPs, ChVNPs either alone or mixed when compared with the control group. The AgNPs-supplemented group had the highest growth performance indicators and lowest FCR compared to other treated groups. The expected weights of tilapia fish after 20 weeks were 195.92, 272.32, 317.39, and 280.16 g. in control, ChVNPs, AgNPs, and mixed groups respectively (Table 3).

Changes in oxidative biomarkers and energy metabolism of hepatic tissues

As presented in Table 4, the CAT levels were significantly increased (p<0.05) in the group supplemented with ChVNPs and the mixed group but significantly reduced (p<0.05) in fish supplemented with AgNPs alone. The MDA, NO, GSSG were significantly decreased (p<0.05) in the mixed group in comparison with other groups, whereas GSH were significantly increased (p<0.05) in the mixed group of

AgNPs and ChVNPs compared to other groups. The hepatic SOD levels were not significantly changed in all dietary treated groups compared to control. Table 4 also demonstrated that ADP and AMP were significantly decreased (p<0.05) in mixed group of AgNPs and ChVNPs compared to other groups, while the ATP was significantly increased (p<0.05) in the mixed group compared to other groups.

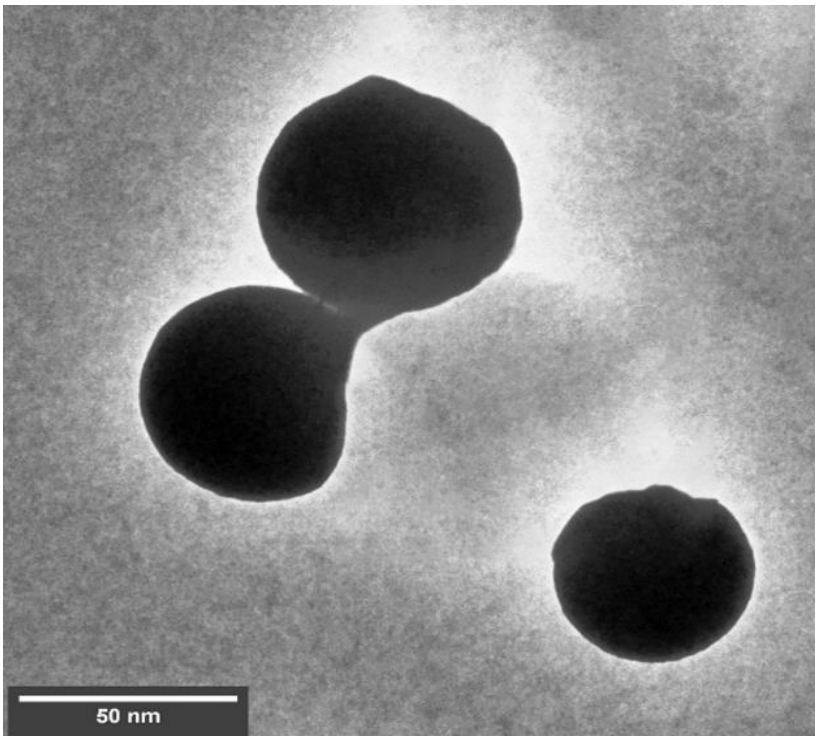

Figure 4: The Size and Shape of ChVNPs determined by TEM that were performed on JEOL JEM-2100 high resolution transmission electron microscope at an accelerating voltage of 200 kV, respectively. The TEM image of ChVNPs at different scales and SAED pattern appeared spherical.

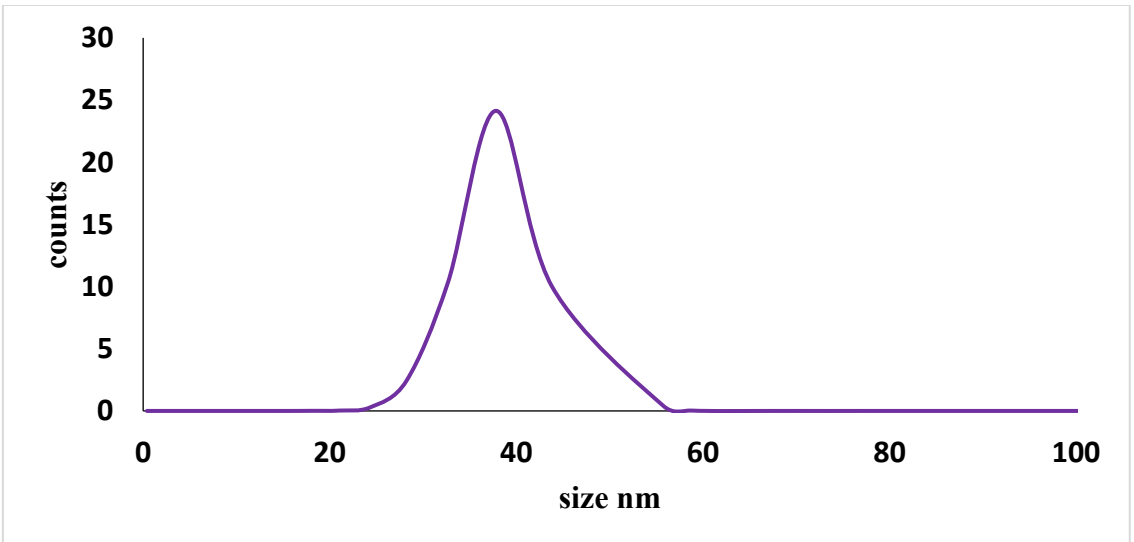

Figure 5: UV-Vis absorption spectra obtained on an Ocean Optics USB2000+VIS-NIR Fiber optics spectrophotometer showing the spectrum of ChVNPs.

Exposure to oxygen and cold stresses induced significant reduction (p<0.05) in hepatic antioxidant biomarkers (SOD, CAT, and GSH) compared to non-stressed fish. The MDA, NO, and GSSG were significantly increased (p<0.05) in fish exposed to oxygen and cold stresses compared to the non-stressed group. Exposure to hypoxia and cold stresses induced a significant decrease (p<0.05) in hepatic ATP and significant increase in ADP and AMP compared to non-stressed fish (Table 4).

Table 3: Changes in growth performance in Nile tilapia supplemented with ChVNPs, AgNPs, and Mixed group for 45 days and the expected performance after 20 weeks.

Items	Control	ChVNPs	AgNPs	AgNPs and ChVNPs (Mixed group)
Growth performance parameters during the First forty-five days				
	Mean ±S.E	**Mean ±S.E**	**Mean ±S.E**	**Mean ±S.E**
Initial weight. (g)	10.03±0.04	10.05±0.06	9.96±0.02	10.19 ± 0.13
Final weight (g)	17.31^{c} ±0.86	24.11^{b} ±0.65	28.13^{a} ±0.77	24.84^{b} ±0.57
Weight gain (g)	7.29^{c} ±0.86	14.07^{b} ±0.65	18.13^{a} ±0.77	14.84^{b} ±0.57
Weight gain rate	72.9^{c} ±8.59	140.06^{b} ±6.48	181.26^{a} ±7.74	148.4^{b} ±5.67
SGR (%)	4.36^{c}	5.81^{b}	6.4^{a}	10.72^{b} ±0.19
Initial length (cm)	9.47±0.2	9.56±0.19	9.45±0.19	9.73±0.19
Final length (cm)	11.05±0.2	11.46± 0.4	11.19±0.3	11.21 ±0.2
Length gain (cm)	1.58±0.41	1.80±0.04	1.64 ±0.04	1.47±0.03
Feed intake (g)	11.82± 0.21	11.78 ±0.32	11.57 ±0.47	11.67 ±0.61
FCR	1.62^{a} ±0.11	0.84^{b} ±0.06	0.64^{b} ±0.07	0.79^{b} ±0.05
Expected Final Weight at 20^{th} week according to Dampin *et al*. (2012)				
Marketing weight	195.92	272.32	317.39	280.16

FCR: Feed conversion ratio; SGR: specific growth rate; ChVNPs: Chlorella Vulgaris nanoparticles; AgNPs: Silver nanoparticles; AgNPs and ChVNPs: Chlorella Vulgaris nanoparticles and silver nanoparticles.
Means with different superscript letters at the same row differ significantly at p<0.05. SEM: Data are presented as means±standard error of the mean.

Table 4: Hepatic oxidant-antioxidant indices and energy metabolism changes in Nile tilapia subjected to hypoxia and cold stress and supplemented with ChVNPs, AgNPs, and combined AgNPs and ChVNPs (Mixed group).

	Control			ChVNPs			AgNPs			AgNPs and ChVNPs				P values		
	No stress	**Hypoxia**	**Cold stress**	**No stress**	**Hypoxia**	**Cold stress**	**No stress**	**Hypoxia**	**Cold stress**	**No stress**	**Hypoxia**	**Cold stress**	**SEM**	**Treatment (T)**	**Stress (S)**	**T*S**
SOD (nM/g)	48.85^{bA}	$41.89b^{B}$	$41.22b^{B}$	47.96^{bA}	$41.69b^{B}$	40.36^{bB}	47.57^{bA}	$40.97bb^{B}$	41.00^{bB}	55.20^{aA}	33.43^{abB}	34.75^{abB}	1.778	0.947	0.035	0.837
CAT (nM/g)	15.53^{bc}	12.87^{cd}	12.87^{cd}	19.50^{ab}	16.77^{bc}	16.29^{bc}	12.90^{c}	11.04^{d}	11.09^{d}	23.10^{a}	19.49^{ab}	20.63^{ab}	0.330	0.000	0.004	0.982
MDA (nM/g)	40.96^{aB}	46.30^{aA}	47.40^{aA}	40.47^{a}	46.03^{a}	46.31^{a}	41.59^{aB}	48.93^{aA}	48.26^{aA}	29.45^{b}	33.32^{b}	34.17^{b}	0.509	0.000	0.000	0.979
NO (nM/g)	0.49^{aB}	0.56^{aA}	0.55^{aAB}	0.48^{aB}	0.55^{aA}	0.55^{aA}	0.50^{aB}	0.58^{aA}	0.59^{aA}	0.40^{b}	0.46^{b}	0.47^{b}	0.005	0.000	0.000	0.945
GSH (nM/g)	4.96^{bA}	4.26^{bB}	4.29^{bB}	4.86^{b}	4.20^{b}	4.16^{b}	$4.87A^{b}$	4.31^{ABb}	4.13^{bB}	7.28^{a}	6.29^{a}	6.37^{a}	0.060	0.000	0.000	0.966
GSSG (nM/g)	0.43^{a}	0.49^{a}	0.49^{a}	0.39^{ab}	0.44^{ab}	0.44^{a}	0.38^{abB}	0.44^{abA}	0.43^{aA}	0.26^{b}	0.29^{b}	0.29^{b}	0.010	0.000	0.094	0.999
ATP (µg/g)	47.69^{b}	40.41^{b}	41.28^{b}	46.53^{bA}	39.71^{bB}	39.87^{bB}	46.64^{bA}	38.76^{bB}	38.87^{bB}	65.12^{aA}	55.36^{aB}	57.54^{aB}	0.556	0.000	0.000	0.990
ADP (µg/g)	24.22^{bcB}	27.44^{abA}	27.47^{abA}	22.96^{cB}	26.47^{abA}	26.50^{abA}	25.48^{bcB}	29.47^{aA}	29.46^{aA}	18.63^{dB}	21.27^{cA}	21.90^{cA}	0.295	0.000	0.000	0.997
AMP (µg/g)	11.79^{ab}	13.76^{a}	13.71^{a}	10.30^{bc}	11.79^{ab}	11.70^{ab}	11.51^{ab}	13.08^{a}	13.17^{a}	8.80^{d}	10.01^{cd}	10.07^{cd}	0.257	0.000	0.029	1.000

ChVNPs: *Chlorella Vulgaris* nanoparticles; AgNPs: Silver nanoparticles; AgNPs and ChVNPs: *Chlorella Vulgaris* nanoparticles and silver nanoparticles; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate: MDA: malondialdehyde; CAT: catalase; SOD: superoxide dismutase; nM: nanomole; MDA: malonaldehyde; GSH: reduced glutathione; GSSG: oxidized glutathione; NO: nitric oxide.
Means with different small superscript letters at the same row differ significantly at p<0.05 for treatment effect.
Means with different large superscript letters at the same row differ significantly at p<0.05 for stress effect.
SEM: standard error of the means.

Changes in plasma cortisol, hepatic function, and lipid profile

The data presented in Table 5 revealed that the reduced DO in Tilapia significantly increased (p<0.05) the plasma cortisol level compared to cold stress and compared to control. Cold stress significantly increased the cortisol than the control non-stressed group but lower than the oxygen stress. Nile tilapia received a diet supplemented with the mixture of AgNPs and ChVNPs significantly decreased (p<0.05) the cortisol level compared to Tilapia treated with each alone and to control.

The data presented in Table 5 showed that the ALT and AST activities were significantly lower (p<0.05) in Tilapia fed with a diet supplemented with the mixture of AgNPs and ChVNPs in comparison with other groups. The lipid profile showed a significant reduction (p<0.05) of TC and TG in Nile tilapia treated with a mixture of AgNPs and ChVNPs compared to other groups.

Table 5: Changes in plasma lipid profile, hepatic function, and cortisol in Nile tilapia subjected to hypoxia and cold stress and supplemented with ChVNPs, AgNPs, and combined AgNPs and ChVNPs (Mixed group).

	Control			ChVNPs			AgNPs			AgNPs and ChVNPs				P values		
	No stress	Hypoxia	Cold stress	No stress	Hypoxia	Cold stress	No stress	Hypoxia	Cold stress	No stress	Hypoxia	Cold stress	SEM	Treatment (T)	Stress (S)	T*S
TC (mmol/L)	2.44a	2.34a	2.42a	2.21ab	2.26ab	2.19ab	2.22ab	2.23ab	2.40a	1.47d	1.64cd	1.55cd	0.66	0.001	0.939	0.993
TG (mmol/L)	0.97a	0.97a	1.03a	0.95ab	0.95ab	1.00ab	0.97ab	0.88ab	1.01a	0.68d	0.69cd	0.75cd	0.033	0.020	0.607	0.999
ALT (U/L)	37.97^{b}	40.00^{ab}	46.93^{a}	38.93^{b}	37.73^{b}	41.20^{ab}	40.20^{ab}	41.30^{ab}	43.23^{ab}	29.83^{c}	29.77^{c}	28.77^{c}	1.408	0.016	0.592	0.969
AST (U/L)	39.30^{ab}	41.73^{ab}	42.90^{a}	42.57^{a}	42.50^{a}	41.80^{ab}	40.23^{ab}	44.87^{a}	41.17^{ab}	25.80^{c}	28.30^{c}	25.70^{c}	1.635	0.005	0.837	0.999
Cortisol (µg/dl)	29.04^{aC}	34.53^{aA}	32.85^{aB}	28.93^{aB}	36.37^{aA}	32.45^{aB}	30.41^{a}	37.29^{a}	35.14^{a}	21.47^{b}	24.64^{b}	23.41^{b}	0.312	0.000	0.000	0.507
TC (mmol/L)	2.44a	2.34a	2.42a	2.21ab	2.26ab	2.19ab	2.22ab	2.23ab	2.40a	1.47d	1.64cd	1.55cd	0.66			
TG (mmol/L)	0.97a	0.97a	1.03a	0.95ab	0.95ab	1.00ab	0.97ab	0.88ab	1.01a	0.68d	0.69cd	0.75cd	0.033			

ChVNPs :Chlorella Vulgaris nanoparticles; AgNPs :Silver nanoparticles; AgNPs and ChVNPs: Chlorella Vulgaris nanoparticles and silver nanoparticles ; TC: Total Cholesterol; TG: Triglycerides; ALT: Alanine amino transferase; AST: Aspartate aminotransferase.
Means with different small superscript letters at the same row differ significantly at p<0.05 for treatment effect.
Means with different large superscript letters at the same row differ significantly at p<0.05 for stress effect.
SEM: standard error of the means.

The highest level of stress biomarkers (Cortisol, TC and and TG) was demonstrated in group feed on AgNPs followed by ChVNPs group and the lowest was in the combined group.

Changes in the amino acid profile of fish muscle

As shown in Table 6, the muscle ARG, HIS, LYS, PHEN, VAL, and ASP were significantly decreased (p<0.05) in the fish treated with the combination of AgNPs and ChVNPs when compared with the control group. The musculature ISO, LEU, METH, TUA, SER, GLY, ALA, and PRO values were significantly higher (p<0.05) in *O. niloticus* received diet supplemented with combination of AgNPs and ChVNPs when compared to other dietary treated groups. The muscle THR and TYR were not affected by the addition of AgNPs and ChVNPs to the diets either alone or in combination. The AgNPs and ChVNPs each alone significantly decreased the muscle GLU when compared to other groups. Exposure to hypoxia stress in

Tilapia significantly reduced (p<0.05) the musculature ISO, LEU, METH, PHEN, and TAU, however; musculature LYS and PRO significantly increased. Cold stress significantly decreased (p<0.05) musculature ISO and METH concentration, while increasing the musculature LYS and PRO values.

Table 6: Changes in muscle amino acid profile (nmol/g meat) of Nile tilapia subjected to hypoxia and cold stress and supplemented with ChVNPs, AgNPs, and combined AgNPs and ChVNPs (Mixed group).

	Control			ChVNPs			AgNPs			AgNPs and ChVNPs				P values		
	No stress	hypoxia	Cold stress	No stress	hypoxia	Cold stress	No stress	hypoxia	Cold stress	No stress	hypoxia	Cold stress	SEM	Treatment (T)	Stress (S)	T*S
Arginine	1.48^{a}	1.59^{a}	1.53^{a}	1.42^{a}	1.45^{a}	1.29^{b}	1.44^{a}	1.39^{ab}	1.56^{a}	1.26^{c}	1.33^{b}	1.28^{b}	0.035	0.119	0.913	0.881
Histidine	0.36^{ab}	0.37^{ab}	0.38^{ab}	0.37^{ab}	0.40^{a}	0.43^{a}	0.35^{ab}	0.38^{a}	0.37^{ab}	0.32^{c}	0.29^{c}	0.31^{c}	0.007	0.001	0.521	0.743
Isoleucine	1.08^{b}	0.88^{b}	1.00^{b}	1.05^{bA}	0.87^{bB}	0.88^{bB}	1.07^{b}	0.89^{b}	0.96^{b}	1.51^{a}	1.28^{a}	1.43^{a}	0.019	0.000	0.001	0.955
Leucine	1.23^{bA}	1.04^{bB}	1.11^{bB}	1.17^{bA}	1.00^{bB}	1.22^{bA}	1.17^{b}	0.99^{b}	1.09^{b}	1.74^{a}	1.52^{a}	1.59^{a}	0.016	0.000	0.000	0.603
Lysine	0.83^{bB}	0.98^{aA}	0.92^{abAB}	0.78^{bcB}	0.92^{abA}	0.89^{bA}	0.80^{bcB}	0.92^{abA}	0.87^{bA}	0.65^{dB}	0.75^{cdA}	0.72^{cdA}	0.015	0.000	0.006	0.998
Methionine	0.16^{b}	0.14^{bc}	0.13^{bc}	0.16^{bA}	0.14^{bcAB}	0.12^{cB}	0.16^{b}	0.13^{bc}	0.15^{bc}	0.23^{a}	0.19^{a}	0.19^{a}	0.003	0.000	0.001	0.700
Phenyl alanine	0.54^{ab}	0.45^{b}	0.45^{b}	0.51^{ab}	0.44^{bc}	0.47^{b}	0.53^{abAB}	0.45^{bB}	0.55^{aA}	0.46^{b}	0.40^{cd}	0.44^{bc}	0.007	0.008	0.001	0.422
Threonine	1.15^{a}	1.11^{a}	1.05^{ab}	1.13^{a}	1.18^{a}	1.07^{ab}	1.15^{a}	1.16^{a}	1.18^{a}	0.92^{b}	0.89^{b}	1.04^{ab}	0.027	0.050	0.998	0.846
Valine	1.14^{b}	1.27^{a}	1.07^{bc}	1.18^{ab}	1.17^{ab}	1.17^{ab}	1.19^{ab}	1.14^{b}	1.12^{b}	1.04^{bc}	0.97^{c}	0.93^{c}	0.020	0.006	0.353	0.687
Taurine	5.70^{b}	4.94^{b}	5.79^{b}	5.54^{bA}	4.63^{bB}	5.31^{bAB}	5.77^{b}	4.90^{b}	5.38^{b}	8.38^{aA}	7.22^{aB}	8.35^{aA}	0.107	0.000	0.004	0.979
Aspartic acid	1.16^{b}	1.21^{ab}	1.27^{a}	1.18^{ab}	1.19^{ab}	1.27^{a}	1.12^{b}	1.04^{bc}	1.07^{bc}	1.09^{b}	1.07^{bc}	0.98^{c}	0.024	0.041	0.950	0.808
Serine	1.18^{b}	1.10^{bc}	0.95^{c}	1.16^{b}	1.21^{b}	1.15^{b}	1.15^{b}	1.30^{ab}	1.18^{b}	1.31^{ab}	1.46^{a}	1.31^{ab}	0.031	0.026	0.309	0.875
Glutamic acid	2.87^{a}	2.84^{a}	2.90^{a}	2.79^{ab}	2.70^{b}	2.39^{c}	2.64^{b}	2.41^{c}	2.49^{bc}	2.78^{ab}	2.85^{a}	2.58^{bc}	0.037	0.014	0.159	0.494
Glycine	0.64^{b}	0.69^{ab}	0.65^{b}	0.63^{b}	0.64^{b}	0.65^{b}	0.63^{b}	0.62^{b}	0.62^{b}	0.90^{a}	0.91^{a}	0.92^{a}	0.015	0.000	0.915	0.998
Alanine	2.02^{cd}	1.80^{d}	1.75^{d}	2.11^{cd}	2.29^{c}	2.12^{c}	2.03^{cd}	1.83^{d}	1.98^{cd}	2.93^{ab}	3.20^{a}	2.70^{b}	0.035	0.000	0.194	0.142
Tyrosine	0.66^{a}	0.67^{a}	0.60^{a}	0.65^{a}	0.56^{ab}	0.53^{b}	0.63^{a}	0.60^{a}	0.64^{a}	0.60^{a}	0.66^{a}	0.56^{ab}	0.014	0.398	0.317	0.683
Proline	0.78^{cB}	0.89^{bcAB}	0.91^{bA}	0.82^{bcB}	0.94^{bA}	0.86^{bcB}	0.78^{cB}	0.91^{bA}	0.91^{bA}	1.12^{a}	1.32^{a}	1.30^{a}	0.016	0.000	0.003	0.899

ChVNPs: *Chlorella Vulgaris* nanoparticles; AgNPs: Silver nanoparticles; AgNPs and ChVNPs: *Chlorella Vulgaris* nanoparticles and silver nanoparticles.
Means with different small superscript letters at the same row differ significantly at p<0.05 for treatment effect.
Means with different large superscript letters at the same row differ significantly at p<0.05) for stress effect.
SEM: standard error of the means.

Effect of AgNPs and ChVNPs on cost and return parameters

The data presented in Table 7 showed that the total returns (TR) of fish supplemented with AgNPs are significantly higher (p<0.05) than other dietary treated groups and control. The total cost was significantly higher in fish supplemented with the combination of AgNPs and ChVNPs followed by ChVNPs, and AgNPs group compared to the control.

Table 7: Costs and return parameters for 100 fish per dollar during the experimental period (45 days).

Items	Control	ChVNPs	AgNPs	AgNPs and ChVNPs
TFC ($)	0.19	0.19	0.19	0.19
Feed cost ($)	1.43^{d}	8.59^{b}	2.86^{c}	10.03^{a}
TVC ($)	2.07^{d}	9.23^{b}	3.50^{c}	10.66^{a}
TC ($)	2.26^{d}	9.42^{b}	3.69^{c}	10.85^{a}
TR ($)	3.31^{c}±0.16	4.6^{b}±0.12	5.37^{a}±0.15	4.74^{b}±0.11

Means within the same row carrying different superscript letters are significantly different (p<0.05).
TFC: Total fixed cost; TVC: Total variable cost; TC: Total cost; TR: Total return.

The data presented in Table 8 showed that fish supplemented with AgNPs had the highest net profit ($ 42.91), followed by the mixed and ChVNPs groups that were $28.64 & $28.58, respectively, while it was the lowest for the control group ($21.15).

Table 8: The expected total return and net profit for 100 fish in the 20th week after adding $14 as TC for all groups.

Items	Control	ChVNPs	AgNPs	AgNPs and ChVNPs
TC ($)	16.26	23.42	17.69	24.85
TR ($)	37.41	52.0	60.6	53.49
NP ($)	21.15	28.58	42.91	28.64

TC: Total cost; TR: Total return; NP: Net profit.

Discussion

Environmental stresses, such as inappropriate temp and reduced dissolved oxygen hinder production and disease control in aquaculture through their adverse effect on physiological function, growth, and survival of fish (Dawood *et al*., 2016). Recently, the use of nanotechnology has been proposed for different aquaculture systems to overcome the adverse effects of environmental stressors (Fajardo *et al*., 2022). It is postulated that the size of nanoparticles plays a critical role in their efficacy in inhibiting microbial growth as nanoparticles with smaller sizes have a better effect, which increases their surface area and enhances their interaction compared to bigger particles (Soleimani and Habibi-Pirkoohi, 2017).

The potential use of nanotechnology would show the way to the progression of smart and high-performing fish (Aamir Hussain *et al*., 2020). In our study, nanoparticles were added only during the first six weeks of rearing to improve the body weight gain during the experimental period and consequently all over the period of aquaculture. Another objective was to reduce the total cost and period of aquaculture to twenty weeks in comparison with the normal cultural period that usually ranged from six to eight months according to the recorded ideal weight (Dampin *et al*., 2012). The results revealed that growth performance and feed utilization of Tilapia fish with nanoparticles supplemented as feed additives, especially AgNPs, were significantly improved compared to control. Vineela *et al*. (2017) found that dietary AgNPs supplemented diet improved the growth of Catla fish. Moreover, Mabrouk *et al*. (2021) reported that dietary supply with a concentration of 0.04 mg/kg diet and 10 µg AgNPs L^{-1} increased growth performances in Nile tilapia. More recently, the dietary inclusion of AgNPs (at 10 and 15 µgKg^{-1} feed) enhances growth, health, and protective immune response against *A. hydrophila* (Ibrahim *et al*. 2022). Additionally, the growth performance, immunity, and antioxidant status were improved in *Labeo rohita* fish fed diets containing AgNPs (10 and 15 g/Kg) (Popoola *et al*., 2023). Moreover, dietary supplementation of tilapia with 1.0 mg AgNPs/kg diet for 60 days augmented the growth performance (Aly *et al*., 2023). On the contrary, Hamed and Abdel-Tawwab

(2021) showed that AgNPs exposure alone significantly suppressed the fish growth, and reduced the elevated blood glucose and cortisol. It is noteworthy that the later study was different from ours as it used a sublethal dose of AgNPs (2 mg/L), which is 100 times the dose of AgNPs we used in our study. Moreover, the later study added the AgNPs directly to the water whereas in our study we added the NPs as a feed additive. Because of this contradiction and to interpret the NPs effect in the right manner, Kumar *et al*. (2018) explained that the right application of dietary AgNPs could reduce biological and chemical stress while improving zootechnical performance, immunological health, and aquatic animal longevity. The improvement of growth performance indicators in our study might be attributed to the antimicrobial activity of AgNPs and their modifying effects on the aquatic microflora. It has been shown that AgNPs could increase the population of beneficial bacteria like lactic acid bacteria in fish intestine (Vadalasetty *et al*., 2018). Regarding ChVNPs effect on growth performance indicators, our results were similar to those reported by Badwy *et al*. (2008) who noted that growth performance and feed conversion ratio were significantly higher in fish feed diets containing 50% of *Chlorella* spp than the control group. Consistent with our findings, a dose-dependent increase was observed in feed intake, specific growth rate, and weight gain (%) in ChV-fed tilapia (Abdel-Tawwab *et al*. 2022). In line with our results, Aly *et al.* (2023) concluded that *chlorella* is an echo-friendly material that enhances growth and promotes immunity in a. Concerning the expected weight at 20^{th} weeks, Dampin *et al*. (2012) recorded that the weight of Nile tilapia was about 195.92 gm at this age, and according to the significant increase in the final weight of our treated groups during the experimental period, we estimated the percentage of the growth rate difference to expect the final weight of our treated groups at 20^{th} weeks, they were 272.32, 317.39 and 280.16 for ChVNPs, AgNPs, and mixed group, respectively. These results indicated that all treated groups had a higher growth rate and reached a marketable weight faster than the control one with superiority to the AgNPs group. An increase in the body weight in the treated group with ChVNPs could be attributed to the high protein content of Chlorella (51-58%) and its content of different essential amino acids, demonstrating that Chlorella could be employed as a protein source for animal diet (Becker, 2007). Also, Xu *et al*. (2014) claimed that C. vulgaris could increase digestive enzymes and results in improving growth performance and immune response. Chlorella possesses a significant concentration of polysaccharides, lipid, minerals, and other bioactive components involved in many physiological activities (Xu *et al*., 2014). The treated group with Ag NPs showed increasing in weight that could be attributed to its activating effect on the population of beneficial bacteria like lactic acid bacteria in the intestine of fish (Vadalasetty *et al*., 2018). Moreover, Mabrouk *et al*. (2021) noted that exposure to AgNPs increased growth performance and decreased FCR in Nile Tilapia with a concentration of 0.04 mg/kg diet and 10 µg Ag NPs, respectively.

In our study, the plasma hepatic functions (ALT and AST) were significantly decreased in Tilapia fingerlings supplemented with a mixture of ChVNPs and AgNPs compared to each alone and compared to the control group. These findings suggest that the integration of AgNPs and ChV had an additive effect on the protection of Nile tilapia liver against different types of physical stresses. The mechanism by which these additives protect the liver could be related to their antioxidant activity (Galal *et al.,* 2018). The use of AgNPs synthesized in combination with plants such as Moringa has been shown to improve the hepatic function in saprolegnia-induced infection in Tilapia (Ibrahim *et al*., 2022).

The results of the current study showed that cold stress and low dissolved oxygen-induced oxidant stress by increasing the hepatic oxidant biomarkers **(**MDA, NO, and GSSG) and reducing the hepatic antioxidant biomarkers (SOD, CAT, and GSH). These results affirmed the occurrence of oxidative stress in Tilapia exposed to hypoxia and cold stresses which is consistent with previous reports (Elabd *et al*., 2020). Consistent with our study, the exposure of Nile tilapia to cold stress enhanced oxidative stress as marked by increased levels of MDA and decreased SOD, CAT, and GSH (Yang *et al*., 2022). Our results showed a significant decrease in MDA, NO, and GSSG and a significant increase in GSH in Tilapia treated with a mixture of AgNPs and ChVNPs compared to other groups. It has been found that incorporating *C. vulgaris* into Nile tilapia diet improved antioxidant status (Mahmoud *et al*., 2020a). In fish, dietary *Chlorella* powder supplementation improves anti–oxidative activity and protects Tilapia against sodium arsenate toxicity and penoxsulam toxicity (Galal *et al*., 2018). In addition, supplementing feeds with 15 g/kg diet of ChV supports the antioxidant-immune responses in cadmium-induced toxicity in Nile tilapia fingerlings (Abdel-Tawwab *et al*., 2023). The antioxidant activity reported in this study could be attributed to the polyphenols and flavonoids present in *Chlorella* (Shibata *et al*., 2003). The data reported here appear to support the assumption that the dietary combination of AgNPs and ChVNPs potentiates the antioxidant activity than the effect of each alone. A recent study showed that the Moringa synthesized silver nanoparticles (MS-AgNPs) an exhibited antioxidant effect in tilapia (Ibrahim *et al*., 2022).

Exposure of Tilapia to hypoxia and cold stresses induced a significant reduction in ATP and a significant increase in ADP and AMP compared to non-stressed fish. Similar results were observed by Pollock *et al*. (2007). The reduction of ATP in stressed fish could be attributed to the exhaustion of ATP energy source to repair tissue proteins (Sharp *et al*., 2013) or the inhibition of the ATPase activity by oxidative stress (Ajima *et al*., 2021). This is supported by the finding of increasing ADP and AMP since ATPases are groups of enzymes responsible for catalyzing the hydrolysis of a phosphate bond in adenosine triphosphate (ATP) to form adenosine diphosphate (ADP). Our results support the hypothesis that supplementation of the diet with ChVNPs and AgNPs together improved the energy stores by increasing the ATP

synthesis in O. *niloticus* since the ATP was significantly increased and the ADP and AMP were significantly reduced in Tilapia treated with a mixture of AgNPs and ChVNPs compared to other groups.

In aquaculture practices, the welfare and growth are adversely modulated by different environmental and nutritional conditions that may represent stress factors for fish (Abd El-Hack *et al*., 2022). Regarding the environmental factors, the optimum range of temperature for the growth of tilapia is 25-28°C (Pandit and Nakamura, 2010), whereas the optimum level of dissolved oxygen is usually greater than 3 mg/L (Abd El-Hack *et al*., 2022). Changing the optimum range of water temp or DO may affect the growth rate or even death of fish (Pandit and Nakamura, 2010). In fact, the ability of water to keep the normal levels of oxygen is dependable on water temperature as the low water temperature reduces the ability of the water to keep DO (Kreger, 2004). The reduced dissolved oxygen significantly increased the cortisol level than that of cold stress and compared to control, a result that coincided with previous reports (Mahmoud *et al*., 2020b). Fatima *et al*. (2021) reported an increase in cortisol levels in *O. niloticus* during October and November (temperature 20ºC) compared to June, July, August, and September. The addition of the ChV and AgNPs significantly reduced the stress effect as indicated by the reduced levels of cortisol. This result agreed with that reported by Elabd *et al*. (2020), who reported that dietary incorporation of *ChV* and *Chlorella glabra* reduced the stress and cortisol levels in Nile tilapia exposed to arsenic toxicity and cold stress, respectively. The hepatoprotective effect of AGNPs could be attributed to their role as anti-inflammatory (Wong *et al*., 2009) and antioxidant (Inbathamizh *et al*., 2013) effect. Reshi *et al.* (2017) Found that AGNPs were able to restore the levels of superoxide dismutase (SOD) and catalase (CAT).

Lipids, including triglycerides (TG) and total cholesterol (TC) are considered sensitive makers for stress in fish (Abarra *et al*., 2017). It is well known that stress factors increase the mobilization of lipids from the liver and other tissues for subsequent utilization to face the stress (Ibrahim *et al*., 2022). The fish with dietary combination of AgNPs and ChVNPs had the lowest values of TG and TC compared to each alone and compared to the control. This result suggests that the incorporation of ChVNPs into AgNPs as a dietary supplement is beneficial to alleviate the stress biomarkers in Nile tilapia. This suggestion is based on our results that demonstrated that the highest level of stress biomarkers (Cortisol, TC, and TG) is reported in Nile tilapia feed on AgNPs followed by ChVNPs, while the lowest levels were recorded in the combined group. In line with our findings, previous studies showed that the addition of some medicinal plants, such as pomegranate to AgNPs in water reduced the stress biomarkers in tilapia when compared to fish exposed to AgNPs alone (Hamed and Abdel-Tawaab, 2021). The findings indicated that exposure to AgNPs alone notably increased stress indicators, such as cortisol levels. It has been demonstrated that exposure to AgNPs can lead to the release of Ag+ into the environment and

finally absorbed by living organisms (Cáceres-Vélez *et al*., 2019). Ag-NPs increase the lipid peroxidation whereas Ag+ increases DNA damage (Massarsky *et al*., 2014) and inhibits the Na(+),K(+)-ATPase activity in rainbow trout (Veinot and Goss, 2012). However, microalgae such as chlorella can activate gut microbiota in fish, leading to the release of short-chain fatty acids and critical amino acids that possess an immunomodulatory effect (Albaqami, 2025).

Fish is a healthy food particularly the protein supply, with one-third of the global population consuming 20% of their protein, especially in developing countries. Therefore, the effect of different types of stress on muscle composition is very important. Moreover, finding effective ways to alleviate and minimize the adverse effect on muscle amino acids, which are the main component of fish musculature, is commercially crucial. We have measured 17 amino acids in fish musculature to determine the effect of reduced dissolved oxygen and cold stress on the quality of muscle protein and ways to reduce the adverse effect of these stresses by incorporating ChVNPs and AgNPs in the diet. Our study demonstrated that exposure of *O. niloticus* to reduced dissolved oxygen modulated the amino acids in fish protein by decreasing the musculature ISO, LEU, METH, PHEN, and TAU and increasing musculature LYS and PRO. Moreover, cold stress significantly decreased musculature ISO and METH while increasing LYS and PRO. Fatima *et al.* (2021) demonstrated significant changes in muscle content of essential and non-essential amino acids over a period of six months from June to November. It seems that environmental stresses adversely affect mostly the essential amino acids of Nile tilapia protein. These amino acid modulations were ameliorated with the combined mixture of ChVNPs and AgNPs. *Chlorella vulgaris* is a good protein source for African catfish and can also substitute fishmeal in catfish diets (Enyidi, 2017). Comparable results (Khan *et al*., 2020) showed that essential amino acid levels were significantly higher in the Nile tilapia received dietary nano-nutrients complex (Vitamins incorporated with chemically synthesized nanoparticles including Fe, Zn, Cu, and Se). They also concluded that the incorporation of various important minerals showed strong synergistic interaction with the basal diet in enhancing amino acid synthesis and growth performance in *O. niloticus*. We detected a significant increase in proline in Tilapia fingerlings exposed to cold stress and oxygen stress. It has been found that exposure of *O. niloticus* to stress of salinity and alkalinity increased the proline amino acid in the fish muscle (Cheng *et al*., 2022). Activation of proline dehydrogenase (PDH) by proposed mechanism variation of proline content of the muscle of fish exposed to ambient salinity and alkalinity. Therefore, the modulation of muscle amino acid content by dietary supplementations of ChVNPs and AgNPs could be related to alteration in the muscle enzymatic activities involved in the synthesis of the amino acids. The mechanisms by which the nanoparticles affect the muscle amino acids of *O. niloticus* musculature need further investigation.

Nanotechnology innovations are still in the laboratory stage, generally incoherent

from the public and areas where they are ultimately being practiced. Consequently, people interact with technological advances merely as customers for their commercialization (Pandey and Jain, 2020). Nanotechnology is relatively still of higher cost, so in our study, we applied the nanoparticles to the fish feed for a short period (six weeks) in the early stages to avoid extra feed costs with obtaining a higher growth rate, immunity, and profits all over the cultural period. Our results concluded that the fish supplemented with AgNPs in its feed achieved the best profit ($ 42.91), followed by ChVNPs and Ag NP (Mixed) group, then ChVNPs group ($28.64 and $28.58, respectively), while was the lowest for the control group ($21.15). However, the ChVNPs were proven to reduce the ambient stress of cold and hypoxia as previously discussed. Therefore, it is recommended to add ChVNPs to AgNPs to alleviate the ambient stress factors, and in this case, the adverse stress effects could be overcome without any losses in the return of the farm. However, this supplementation increases the cost, which may reduce net profit in aquaculture farms, but still more profitable than the control one.

Finally, there are still a lot of concerns about using nanotechnology in aquaculture that need more studies to explore the optimum dose and duration of application to produce nontoxic, ecologically friendly and economic products considering the bioaccumulation and environmental outputs (Okeke *et al*., 2022). In addition, the process of production of nanoparticles and its application in aquaculture should be under legislative control to ensure eco-safety (Abbas *et al*., 2021). We did not deeply study these concerns of nanoparticle applications in our study, therefore intensive investigations are required to declare their biological effects on large-scale aquaculture.

Conclusions

In conclusion, the dietary combination of AgNPs and ChVNPs is valuable in the aquaculture of *O. niloticus* to palliate the adverse effect of physical stress caused by hypoxia and cold, promote the hepatic antioxidant process and hepatic functions, restore hepatic energy resources, and modulate muscle amino acid profile. The AgNPs in diet with low concentrations (0.02 mg/kg diet) are more effective in growth performance and more profitable. However, the combined mixture of ChVNPs (5 g/ kg diet) and AgNPs (0.02 mg/kg diet) is recommended to overcome the unexpected consequences of using the mineral nanoparticles alone. Further investigations are still required to explore the safe use of nanoparticles in aquaculture for the purpose of improving growth and fish muscle quality regarding the effective dosing, the optimal durations, and the environmental effects, especially during stress exposure arising from climatic changes.

Conflicts of interest

All authors declare that they do not have any conflict of interest.

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