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A comparative evaluation of sublethal effects of chemical and green synthesized ZnO nanoparticles on histology of *Oreochromis niloticus* (Nile tilapia)

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Abstract
Aquaculture is one of the most important food production industries, both economically and in terms of food security for the world's growing population. *O. niloticus* is the second largest freshwater species that is used as a bio-indicator. Zinc oxide nanoparticles (ZnO NPs) are commonly eluted from industries and excessively accumulate in aquatic environments highly vulnerable to fish that induce alterations in tissues. *C. forskaolii* Vahl is an edible annual herb with high therapeutic value. In the present study, we synthesized green nanoparticles using *C. forskaolii* Vahl acts as a reducing, stabilizing, and capping agent which is an alternative to the conventional chemical method. Further, the comparative evaluation of chemical and green synthesized ZnO NPs exposure on acute toxicity (96hrs), sublethal toxicity (28 days) and histological alterations were examined in the gills, liver and muscles of female *O. niloticus.* The results of acute toxicity of female *O. niloticus* exposed to chemical and green synthesized ZnO nanoparticles were identified to be 2.533 mg/L and 4.210 mg/L. In addition, sublethal toxicity was also found after 28days exposure to chemical and green synthesized ZnO nanoparticles in female *O. niloticus* is 0.168 mg/L, 0.253 mg/L, 0.506 mg/L followed by 0.280 mg/L, 0.421 mg/L and 0.842 mg/L. Furtherly, chemically synthesized ZnO NPs exhibited severe histological alterations such as congestion in gills, congestion of the central vein and hepatic sinusoid with fatty changes of hepatocytes in the liver and congested blood vessels in muscle than in green synthesized ZnO NPs. In conclusion, the present study determined that green synthesized ZnO NPs using *C. forskaolii* Vahl reduce the damages caused by the nanoparticles to aquatic foremost organism fishes compared to the chemical method.

Keywords: nile tilapia (*O. niloticus*), acute toxicity, sublethal toxicity, zinc oxide, nanoparticle, histopathology, *C. forskaolii* vahl

Introduction

Aquaculture is one of the most significant food production sectors that has contributed to food security in recent decades by fulfilling the enormous demand for animal protein for the growing human population all over the world. The second largest aquatic commercial freshwater species *O. niloticus* used as a bio-indicator and model organism for toxicological research due to its adaptability to various environmental changes. Chemical toxins are the main cause of fish mortality in aquaculture and one of the biggest problems with health and well-being. Toxicity refers to the degradation or loss of cell viability and regenerative capability that may arise from the reversible or irreversible disruption of normal metabolic processes. In severe circumstances, the body may lose all of its organs responsible for absorption and elimination leading to death (Caruso *et al*., 2020) [9].

Fish have been discovered to be contaminated with several toxicants including nanoparticles, heavy metals, chemicals, pollution, pesticides, fungicides, algaecides, microplastics, herbicides, chemical fertilizers, plasticizers, chemotherapeutic agents, radioactive materials, residues from extractive industries, pharmaceuticals of human and veterinary medicine are considered the most significant form of pollution in the aquatic fauna and their environment. This leads to a decline in water quality and the rise of infectious diseases like jaundice, diarrhoea, and dysentery (Hampel *et al*., 2015; Bhat *et al*., 2017) [16, 7].

Nanotechnology is one of the most demanding and rapidly expanding science and engineering disciplines. The most competent technology can play a significant role in almost all recent research areas and has broad applicability (Amerasan *et al*., 2016) [5]. Zinc oxide nanoparticles (ZnO NPs), one of the most often utilized nanoparticles, are the world's third most manufactured metal oxide on an industrial scale. Additionally, they will undoubtedly be employed more frequently in various industries due to their special advantages in industrial and therapeutic items. The direct and indirect emissions of nanoparticles into aquatic habitats through sewage effluent and engineering applications will further increase the exposure of ecosystems and humans to nanoparticles. The aquatic fauna is particularly vulnerable to the excess ZnO NPs discharged into the environment. The harmful effects of metal ions are more prone to affect fish (Santhoshkumar *et al*., 2017) [37].

Nanoparticle toxicity in fish easily affects and naturally accumulates in primary organ gills and transfers to other body organs through the circulatory system leading to morphological and functional alternations. In addition, higher concentrations of ZnO NPs can also have harmful effects, disrupt growth promotion, impair reproductive efficiency, increase oxidative stress, induce ROS production, impair metabolism, exhibit bioaccumulation in many fish organs, behavioural changes, and disturb immune system and homeostasis (Chupani *et al*., 2017; Estrela *et al*., 2021) [10, 13].

In recent years, chemical and biological processes have been used to synthesize ZnO NPs. The drawbacks of synthesizing ZnO NPs chemically often include high energy requirements, low purity, irregular particle size distribution, high costs, significant secondary waste production, and irreversible environmental impact (Darroudi *et al*., 2014) [11]. The plant extract-based biogenic synthesis of nanomaterials is still a popular method that is cost-effective, non-toxic, biodegradable in nature, readily available, contamination-free, safe to handle and can easily scale up for large-scale synthesis, eco-friendly approach for nanomaterial production and also have excellent polydispersity, dimensions, stability. Further, the plant contains a diverse range of secondary metabolites that have active functional organic groups that work as reducing, stabilizing, and capping agents, which are important in the green method of nanoparticle synthesis (Jagtap and Bapat, 2013) [19].

Commelina forskaolii Vahl (*C. forskaolii* Vahl) is an edible annual herb belonging to the family Commelinaceae. They are chiefly found as weeds of cultivated ground or along roadside ditches and swamps. The leaves of *C. forskaolii* Vahl are edible as a vegetable. This plant has different ethnomedicinal properties, including indigestion, smoothening of sores, feed for goats and cattle, and the whole plant to make a charm that is used during tribal cleansing rituals (Abbas *et al*., 2020; Poornima and Jeyam, 2016) [1, 31].

Hence, the present investigation was carried out to determine the lethal concentration (LC_{50}) and compare the sublethal effects of chemical and green synthesized ZnO NPs on histological changes in female *O. niloticus*.

Materials and Methods

Collection and Identification of Plant Material

Fresh leaves of *Commelina forskaolii* Vahl (*C. forskaolii* Vahl) were collected from the Coimbatore district, Tamilnadu, India. The plant was taxonomically identified and authenticated by the Botanical Survey of India, Coimbatore, Tamilnadu. The voucher specimen was retained in our laboratory for further reference (Voucher ID: BSI/SRC/5/23/2021/Tech).

Chemical Synthesis of ZnO NPs

ZnO nanoparticles were chemically synthesized by direct precipitation method using zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) and potassium hydroxide (KOH) as precursors. In this work, the aqueous solution (0.2 M) of $Zn(NO_3)_2.6H_2O$ and the solution (0.4 M) of KOH were prepared with deionized water, respectively. The KOH solution was slowly added to the zinc nitrate solution at room temperature under vigorous stirring, resulting in a white suspension formation. The white product was centrifuged at 5000 rpm for 20 min and washed three times with distilled water, and washed with absolute alcohol at last. The obtained product was calcined at 500°C in an air atmosphere for 3 hrs (Ghorbani *et al*., 2015) [14].

Green Synthesis of ZnO NPs

ZnO nanoparticles were successfully synthesized using *C. forskaolii* Vahl plant leaves extracts by adopting a procedure with slight modifications (Rajiv *et al*., 2013) [32]. Prepared 0.1M of zinc nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$) in 90ml of double-distilled water, and then 10ml of *C. forskaolii* Vahl leaves extract was slowly added dropwise into the solution under magnetic stirring at 60°C for roughly 4h to obtain a brown suspension formation. The complex formed after stirring was collected by centrifugation at 10,000rpm for 10min. Then, the centrifuge complex was rinsed with water, subjected to centrifugation at 5000rpm for 20min and washed twice with distilled water followed by centrifugation. The separated complex was dried in an oven at 40°C for 8h and was then calcined in a muffle furnace at 100°C to obtain green synthesized ZnO nanoparticles.

Experimental Animals

Two hundred and seventy female Nile Tilapia (*O. niloticus*) were collected from Valankulam Lake, Ukkadam, Coimbatore district, Tamil Nadu, India, and acclimated in the laboratory for two weeks in a fish tank with 500 L aerated dechlorinated tap water. All female fish weighing about 170±5g; with lengths of 22.5 to 23.5 cm were used throughout this study to have homogeneity of the sample. During the entire study period, fish were fed with commercial fish feed which is available in the market.

Acute Toxicity (LC_{50})

Fish were exposed to chemically and green synthesized ZnO NPs for 24, 48, 76, and 96 hrs. The Nile tilapia were exposed to chemically and green synthesized ZnO NPs in various concentrations such as 0 (control), 2, 4, 6, 8, and 10 mg/L respectively for a period of 96 hrs. All experiments were carried out in 80L plastic tanks with ten fish in each concentration. Mortality was monitored throughout the experiment.

Sub-lethal Toxicity Analysis

Sub-lethal concentrations of chemically and green-produced ZnO NPs on *O. niloticus* were estimated using the fatal concentration of 1/10 values. After getting the middle concentrations and the lower and upper concentrations were calculated.

Plastic tanks with a size of 80 L were used, and each plastic tank was filled with 70 L of water and the triplicate of an experiment was conducted. In each plastic tank, 10 healthy fish were introduced, and different concentrations of chemically synthesized ZnO NPs (0.168 mg/L, 0.253 mg/L and 0.506 mg/L) were added, whereas the control was kept without ZnO NPs. Green synthesized ZnO NPs were subjected to varying concentrations (0.280 mg/L, 0.421 mg/L and 0.841mg/L) in each plastic tank. Fish' manifestation and survival times were studied in each concentration for 28 days. The medium of the aquaria was renewed daily and fresh solutions were spiked to maintain water quality. Throughout the period of exposure, fish were closely observed and clinical signs were monitored.

The fish's behaviour was observed in all exposures, including the control and throughout the experiment, sufficient aeration was maintained. After the experiment, the fish are sacrificed for histological study.

Histology

The liver, muscle and gills tissues from the control, chemical and green synthesized ZnO nanoparticles using *C. forskaolii* Vahl groups were removed and fixed with formalin (10%) for histopathological analysis. Hematoxylin and eosin stain was used to stain the tissues. After that, the

slides were examined under a microscope (Pearse, 1968; Roberts, 1978; Humason, 1979). [30, 35, 15]

Statistical Analysis
Statistical Probit analyses were performed using SPSS to determine the LC_{50} of the chemical and green synthesized ZnO NPs.

Results
LC_{50} Analysis
The present results observed that acute toxicity tests for chemical and green synthesized ZnO NPs are presented in Table 1. During the experiment, there was no mortality in the control group. In all treatments, chemically synthesized ZnO NPs showed mortality during 24, 48, 72 & 96 hours, and the median lethal concentration was recorded as 13.747 mg/L, 7.541 mg/L, 4.479 mg/L and 2.533 mg/L respectively. No mortality was observed on the first day (24 hours) of green synthesized ZnO NPs, and all fish in the experimental tanks were healthy. The median lethal concentration of green synthesized ZnO NPs of 48, 72 & 96 hours of treatment was found as 11.616 mg/L, 8.686 mg/L and 4.210 mg/L respectively. Fish mortality increased significantly when the concentration of ZnO NPs (chemical and green synthesized) and the duration of exposure were increased. The data on probit regression/log concentration of *O. niloticus*, when exposed to various concentrations of chemical and green, synthesized ZnO NPs at 96hrs.

O. niloticus exhibited improper behavioural responses that were observed in both chemical and green synthesized ZnO NPs with lethal toxicity. Before death, fish exhibited aberrant swimming movement with a jerking motion, restlessness, a dark discoloration of the skin, loss of equilibrium, breathing difficulties, and incapability to stand upright.

Table 1: Lethal Concentration Determination (LC_{50}) in *O. niloticus*

Time (hrs)	95% confidence limits for concentrations (mg/L)	
	Chemical synthesized ZnO NPs	Green synthesized ZnO NPs
	LC_{50} (mg/L)	LC_{50} (mg/L)
24	13.747	NOEC
48	7.541	11.616
72	4.479	8.686
96	2.533	4.210

NOEC - no- observed – effect concentration

Sublethal Toxicity Analysis
Based on median lethal concentration (LC50), sub-lethal concentrations were calculated as 1/10 values in the chemical (0.253 mg/L) and green (0.421 mg/L) synthesized ZnO NPs on *O. niloticus*. After getting mid concentrations, the lower (1/15 X mid concentration) and upper (1/5 X mid concentration) concentrations were calculated. Totally three different concentrations (Control- 0, CS1-0.168, CS2-0.253, CS3-0.506 and GS1-0.280, GS2 -0.421 and GS3-0.841 mg/L respectively) were maintained for a period of 28 days (Table 2).

Table 2: Sublethal toxicity analysis in *O. niloticus*

Concentration	Nanomaterials (mg/L)	
	Chemical synthesized ZnO NPs	Green synthesized ZnO NPs
Control	0	0
$1/15^{th}$	0.168	0.280
$1/10^{th}$	0.253	0.421
$1/5^{th}$	0.506	0.842

Histological Investigation
The histological analysis revealed that chemical and green synthesized ZnO NPs exposure caused tissue-level changes in targeted organs in *O. niloticus* (gills, liver and muscle).

Gills
Histological analysis of gills of chemical (CS) and green synthesized (GS) ZnO NPs on *O. niloticus* are presented in Plate 1. The gill tissues of the control group exhibited normal morphological arrangements of the lamella, primary and secondary gill lamella with no pathological alterations recorded. In addition, congestion was observed in all ZnO NPs (chemical and green) treated groups when compared to control fish.

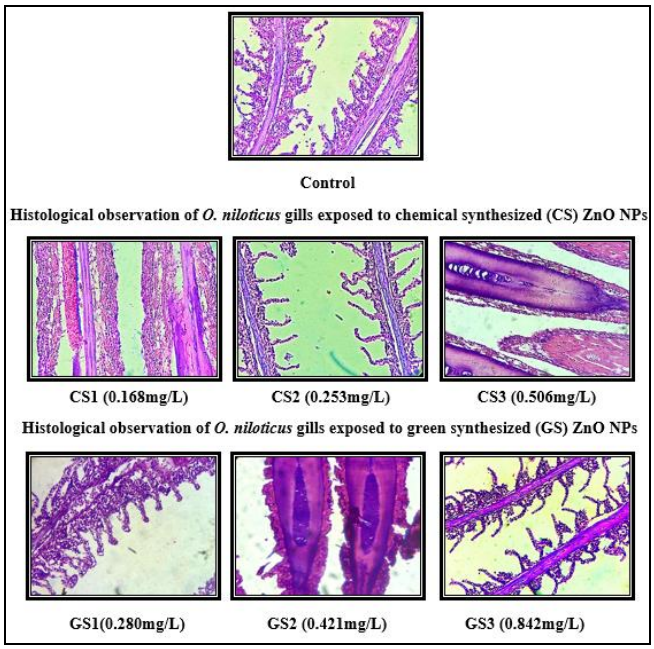

Plate 1: Histological observation of *O. niloticus* gills

Liver
The histoarchitecture of the liver in the control, chemical (CS) and green synthesized (GS) ZnO NPs treated groups is depicted in Plate 2. The control liver showed normal-appearing hepatocytes arranged in lobules with no changes. Numerous alterations were observed in the liver tissues of fish subjected to chemical synthesized ZnO NPs, including severe congestion of the central vein and hepatic sinusoid with fatty changes of hepatocytes while the hepatic tissues of fish treated with green synthesized ZnO NPs showed less damage than chemical synthesized ZnO NPs indicating significant modifications in experimental groups, including normal liver parenchyma with the feature of congestion of sinusoids and central vein at higher concentrations (0.842 mg/L).

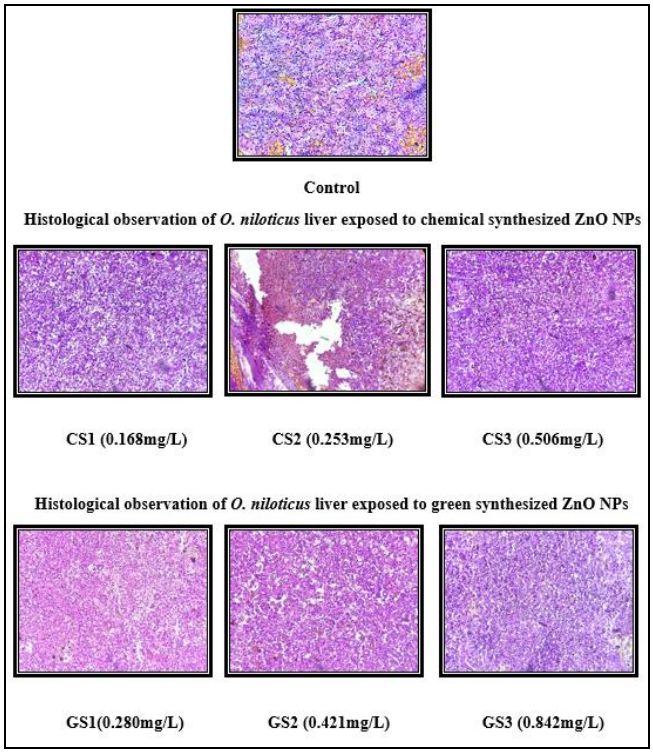

Plate 2: Histological observation of *O. niloticus* liver

Muscle

Histology of muscle in *O. niloticus* exposed to chemical (CS) and green synthesized (GS) ZnO NPs is represented in Plate 3. The muscle tissue of the *O. niloticus* control group was analyzed and observed as normal-appearing myocytes arranged in bundles separated by fibrous septa with no changes in muscle architecture. Normal-appearing myocytes with few congested blood vessels in between were seen at a higher and lower concentration in muscle tissues of chemical and green synthesized ZnO NPs. In higher concentrations of green synthesized ZnO NPs treated (0.841 mg/L), fish exhibited muscle with normal-appearing myocytes in bundles compared to chemically synthesized ZnO NPs.

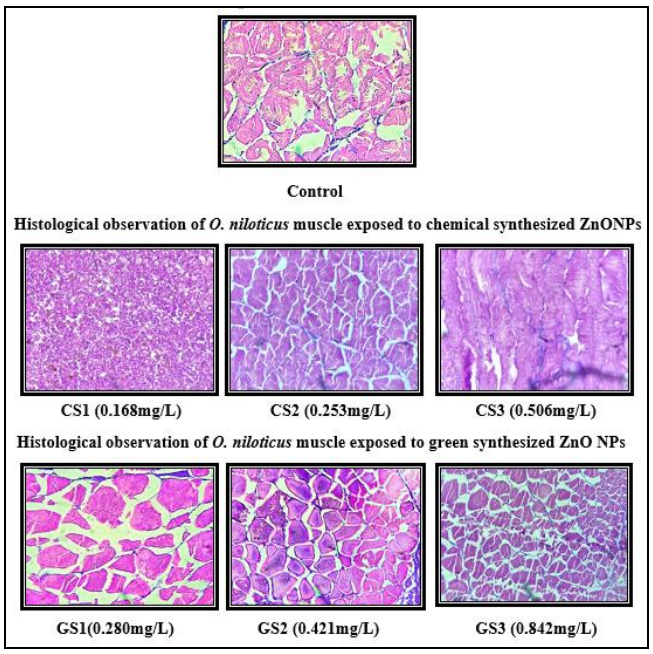

Plate 3: Histological observation of *O. niloticus* muscle

Discussion

Synthesis of ZnO Nanoparticles

The current study discovered that the chemical and green synthesized ZnO NPs are white and brownish in colour, respectively. This result was supported by the findings of Devasenan *et al.*, (2016) [12] the ZnO NPs powder was brownish-green in colour using *Andrographis paniculata* Leaf Extract. Similarly, Joel & Badhusha (2016) [21] observed that green-synthesized ZnO NPs were brown in colour.

Lethal Concentration (LC_{50}) of ZnO NPs

The calculation of LC_{50} values in fish is a highly useful method for assessing the tolerance and safety levels for a certain contaminant or pollutant. The longer the exposure duration, the lower the concentration of ZnO NPs required to kill 50% of the fish population. In addition, the continuous accumulation of a toxin in the fish body enhanced the unfavourable effects within the 4 days of the trials and resulted in a reduction in the LC_{50} value (Moghaddam *et al.*, 2015) [27].

According to Alkaladi *et al.*, (2020) [2] the 96-h LC_{50} value for ZnO NPs was reported to be 4.1 µg/l in female juvenile *O. niloticus*. Previously, Morgalev *et al.*, (2018) [28] also reported 96-h LC_{50} for ZnO NPs as 30.51 mg/L were hazardous to *Danio rerio* (Zebrafish) embryos. Additionally, the LC_{50} 96h value of chemically produced ZnO NPs in rainbow trout (*Oncorhynchus mykiss*) was 0.75 mg/L (Khodabakhshi *et al.*, 2017) [24]. Taherian *et al.*, (2019)[41] stated the acute toxicity of ZnO NPs synthesized from *Satureja hortensis* plant on *Oncorhynchus mykiss* and reported a 96 h LC_{50} value was 25.50 mg/L.

Further, Saddick *et al.*, (2017) [36] noticed the acute toxicity of ZnO NPs on adult *O. niloticus* and *Coptodon zilli* (Red belly tilapia) demonstrating that ZnO NPs are acutely toxic to Nile tilapia with 96hrs LC_{50} concentration of 5.5 mg/L and 5.6 mg/L. However, Aziz *et al.*, (2020) [6] studied the acute toxicity of ZnO NPs in *Labeo rohita* adults and reported 31.15mg/L as a 96-h LC_{50}.

Histological Examination

Histological alterations caused by fish responses to harmful substances in the water and substrate. Histological alterations vary depending on the type of nanoparticles, their concentration, fish species, and the duration of time exposed to nanoparticles, among other factors. Histopathology can assess cellular alterations in organs caused by nanoparticles (Shobana *et al.*, 2018) [40].

Histology of Gills

Gill is a primary target organ for researching the toxicity of chemicals, particularly nanomaterials, on fish and other aquatic species. Nanoparticles mostly enter the fish's body through the gill (Handy *et al.*, 2008) [17]. Apart from respiration, gills perform numerous critical activities such as acid-base balance, ion management, and nitrogenous waste disposal from the animal body. Because of exposure to numerous environmental toxins, some critical and vital organs of the body are impacted directly or indirectly, affecting the health of fish (Nagaraju *et al.*, 2017) [29].

According to a recent report, Khan *et al.*, (2022) [23] observed histopathological abnormalities in the gills of ZnO NPs, ZnO, and a mixed solution of both ZnO NPs and ZnO exposed *O. mossambicus* fish included disorganization of

gill lamellae, cartilaginous core disruption, lifting of epithelium, loss of secondary gill lamellae, blood congestion, a fusion of secondary gill lamellae, shortening of secondary gill lamellae, atrophy, and curling.

Previous research found inflammation, epithelial hyperplasia, and epithelial lifting in the gills of fish fed with chemically synthesized ZnO NP feed (Kurian and Elumalai, 2021) [26]. The gills of *O. niloticus* treated with ZnO NPs displayed extensive vacuolation and necrosis pavement, as well as epithelial cells with dilated mucous cells (Alkaladi *et al.,* 2014) [3]. These pathological abnormalities in the gills can allow toxic compounds to come into direct contact with the fish's circulatory system, compromising respiration and osmoregulatory functions and leading to fish death (Krishnaraj *et al.,* 2016) [25].

Histology of Liver

Hepatic histological alterations are commonly assessed in toxicological studies and are regarded as an indicator of environmental pollution. The liver is a detoxifying organ, as it converts harmful compounds received by the body into non-poisonous substances. As a result of this detoxification effort, the liver accumulates many toxic substances, altering the physiology and histology of hepatocytes. The results obtained also coincide with the reports of Kurian and Elumalai., (2021) [26] that the liver segment of a fish fed with a diet containing chemical synthesized ZnO NPs showed moderate congestion in the blood sinusoids and green synthesized ZnO NPs observed normal hepatic architecture in *O. niloticus*. In addition, previous research has shown that condensed nuclear bodies and pyknotic nuclei were seen along with necrosis and apoptosis in the liver of *O. mossambicus* exposed to ZnO NPs (Shahzad *et al.,* 2019) [39]. Similarly, Kaya *et al*., (2016) [22] observed fatty changes, hepatocellular vacuolations, mononuclear cell infiltrations, pyknotic nuclei and oedema in the liver of *O. niloticus* after a 14-day exposure to both small and large ZnO NPs.

Histology of Muscle

Rajkumar *et al.,* (2022) [34] discovered that biogenic ZnO NPs induced exposure alters histopathological alterations in *C. carpio's* muscles including necrotic muscle fiber and pyknotic nuclei. Moreover, degeneration of muscle bundles, infiltration of inflammatory cells, focal areas of necrosis, splitting of muscle fibers, fractured myofibrils, thickening of muscle bundles, displaced striated muscles, and shortening of muscle bundles were also observed in muscle tissues of African catfish (*Clarias garepinus*) fish exposed to AgNPs (Sayed *et al.,* 2020) [38].

Earlier research by Jayaseelan *et al.,* (2014) [20] on *O. mossambicus* skin exposed to Ni NPs revealed muscle bundle degeneration, focal areas of necrosis, vacuolar degeneration in muscle bundles, edema between muscle bundles, and splitting of muscle fibers. Further, the skeletal muscle fibers in the muscle displayed mild hyalinization, loss of interstitial fibers between the muscle fibers, focal degeneration and necrosis and a minimal infiltration of inflammatory cells after exposure to copper against *Cyprinus carpio* L (Al-Tamimi *et al.,* 2015)[4].

The previous report of Rajkumar *et al.,* (2016) [33] noticed the Ag NPs exposed to *L. rohita* fish displayed abnormal muscle bundle arrangement and vacuolization in muscle. The different authors interpreted the histological changes in the muscle tissue of fishes after being treated with various nanoparticles including heavy metals exposure against *Danio rerio* (Bhuvaneshwari *et al.,* 2015) [8], silver nanoparticles against *O. mossambicus* (Govindasamy *et al.,* 2012) [15].

Conclusion

In the current study, we explored the comparison of chemical synthesized ZnO NPs by precipitation method whereas green synthesized ZnO NPs using aqueous extract of *C. forskaolii* Vahl leaves. Overall the present investigation, suggests chemical and green synthesized ZnO NPs showed different histological alterations in gills, liver and muscles were assessed at the end of sublethal toxicity. In addition, plant-based synthesis of ZnO NPs reduces the histological damages due to the presence of enormous photo-components which can be safe, environment and eco-friendly, low-cost and enhance fish growth. However, further research is required to evaluate the plant-based synthesis of ZnO nanoparticles on various fish models and other aquatic organisms and different applications in the aquaculture and fisheries sector.

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Conflict of Interest

We declare that all authors have no conflict of Interest

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