RESEARCH
www.commprac.com
ISSN 1462 2815

BEHAVIOURAL ANALYSIS OF BETA-CHITOSAN DERIVED COPPER NANOPARTICLES ON STRESS INDUCED ZEBRAFISH

Sarthak Juneja [1], K. Yuvaraj Babu [2], Taniya M [3], M Sundaram K [4]*

[1,2,3,4] Department of Anatomy, Saveetha Dental College and Hospital,
Saveetha Institute of Medical and Technical Science (SIMATS),
Saveetha University, Poonamalle High Road, Velappanchavadi, Chennai.
*Corresponding Author Email: meenakshisundaram.sdc@saveetha.com

DOI: 10.5281/zenodo.12662312

Abstract

This study investigates the behavioral effects of β-chitosan-derived copper nanoparticles (Cu NPs) on stress-induced zebrafish (*Danio rerio*). Zebrafish, a well-established model organism for neurobehavioral studies, were subjected to a chronic unpredictable stress (CUS) protocol to induce stress responses. Following stress induction, the zebrafish were treated with β-chitosan-derived Cu NPs to assess potential anxiolytic and neuroprotective effects. Behavioural assessments were conducted using the novel tank diving test, open field test, and social interaction test to evaluate changes in anxiety-like behavior, locomotor activity, and social behavior. Preliminary results indicate that β-chitosan-derived Cu NPs significantly reduce anxiety-like behavior, as evidenced by increased time spent in the upper zones of the novel tank and increased exploratory behavior in the open field test. Additionally, treated zebrafish displayed enhanced social interaction compared to stressed controls. These findings suggest that β-chitosan-derived Cu NPs possess potential therapeutic properties for mitigating stress-induced behavioral disturbances in zebrafish, potentially translating to broader applications in stress-related neurobehavioral disorders. Further molecular and biochemical analyses are warranted to elucidate the underlying mechanisms of these observed effects.

Keywords: β-Chitosan-Derived Copper Nanoparticles, Zebrafish, Chronic Unpredictable Stress, Anxiety-Like Behaviour, Novel Tank Diving Test, Open Field Test, Neuroprotection, Stress-Induced Behavioural Disturbances.

1. INTRODUCTION

Nanotechnology has emerged as a pivotal field with extensive applications in medicine, biology, and environmental sciences(Nath and Banerjee 2013). Among the various nanomaterials, metal nanoparticles have garnered significant attention due to their unique properties and potential therapeutic benefits(Ambika, Manojkumar et al. 2019, Sahu, Ratre et al. 2021).

Copper nanoparticles (Cu NPs) have been particularly studied for their antimicrobial, antioxidant, and anti-inflammatory properties. Recent advancements in nanoparticle synthesis have explored the use of natural polymers, such as chitosan, to enhance the biocompatibility and stability of these nanoparticles.

In this context, β-chitosan, derived from the shells of crustaceans, has been utilized to synthesize Cu NPs, yielding β-chitosan-derived copper nanoparticles with promising biological activities(Marunganathan, Kumar et al. 2024).

The behavioural analysis of these nanoparticles is crucial to understand their potential neuroprotective and therapeutic effects, especially in stress-related conditions. Zebrafish (*Danio rerio*), a small freshwater fish, has emerged as an ideal model organism for studying neurobehavioral responses due to its genetic similarity to humans, transparent embryonic development, and well-characterized behavioural repertoire(Acharya and Pal 2020, Sahu, Ratre et al. 2021).

Zebrafish are extensively used in neuropharmacology and toxicology research, making them a suitable model for studying the effects of β-chitosan-derived Cu NPs on stress-induced behavioural changes(Tayyeb, Priya et al. 2024). Stress is a natural physiological response to external stimuli, but chronic stress can lead to various neurobehavioral disorders, including anxiety and depression.

Chronic unpredictable stress (CUS) is a widely accepted model for inducing stress in zebrafish, mimicking the unpredictable nature of stressors in human life(Siddiqui, Al-Whaibi et al. 2015). The CUS protocol involves exposing zebrafish to a series of random and varying stressors over an extended period, leading to consistent and measurable stress responses. Behavioural manifestations of stress in zebrafish include increased anxiety-like behaviours, reduced exploratory activity, and altered social interactions(Zahin, Anwar et al. 2020, Sundaram, Bupesh et al. 2022).

Anxiety-like behaviours in zebrafish are typically assessed using the novel tank diving test and the open field test. In the novel tank diving test, zebrafish initially exhibit a preference for the bottom of the tank, reflecting their anxiety levels. Over time, reduced anxiety is indicated by increased exploration of the upper zones. The open field test measures locomotor activity and exploratory behaviour, with decreased activity and reduced center exploration indicating heightened anxiety. Social interaction tests further evaluate the impact of stress on social behaviours, as zebrafish are naturally social animals, and stress can lead to social withdrawal or aggression.

The synthesis of β-chitosan-derived Cu NPs involves reducing copper ions in the presence of β-chitosan, which acts as both a stabilizing and capping agent(Harishchandra, Pappuswamy et al. 2020, Ravikumar, Marunganathan et al. 2024). This green synthesis approach enhances the biocompatibility of the nanoparticles, making them suitable for biological applications. The therapeutic potential of β-chitosan-derived Cu NPs in stress-induced behavioural disorders may be attributed to several mechanisms. Copper is an essential trace element involved in numerous physiological processes, including neurotransmitter synthesis, antioxidant defence, and immune function.

The incorporation of copper into nanoparticles may enhance its bioavailability and therapeutic efficacy. β-Chitosan, known for its biocompatibility and bioactivity, may further augment the effects of Cu NPs by facilitating their cellular uptake and interaction with biological systems. One potential mechanism is the antioxidant activity of β-chitosan-derived Cu NPs.

Chronic stress is associated with increased oxidative stress, which can damage neurons and impair brain function. The antioxidant properties of Cu NPs may help mitigate oxidative damage, thereby protecting neural tissues and preserving cognitive function. Additionally, the anti-inflammatory properties of Cu NPs may reduce neuroinflammation, a common consequence of chronic stress, thereby alleviating behavioural symptoms(Ali, Ghazy et al. 2018, Ponmanickam, Gowsalya et al. 2022).

Another mechanism could involve the modulation of neurotransmitter systems. Copper is a cofactor for several enzymes involved in the synthesis and metabolism of neurotransmitters such as dopamine, norepinephrine, and serotonin(Pandiyan, Sri et al. 2022). By enhancing the availability of copper, β-chitosan-derived Cu NPs may help restore the balance of these neurotransmitters, leading to improved mood and reduced anxiety-like behaviours(Pandiar, Ramani et al. 2022). The experimental design for assessing the effects of β-chitosan-derived Cu NPs on stress-induced zebrafish involves several key steps.

Initially, zebrafish are subjected to the CUS protocol for a specified duration to induce stress responses(Chakravarty, Reddy et al. 2013). Following the stress induction, the zebrafish are treated with β-chitosan-derived Cu NPs at various concentrations to determine the optimal therapeutic dose. Control groups include non-stressed zebrafish, stressed zebrafish without nanoparticle treatment, and zebrafish treated with a vehicle solution.

Behavioural assessments are conducted at multiple time points to evaluate the effects of the nanoparticles on anxiety-like behaviour, locomotor activity, and social interaction(Wu, Yan et al. 2023). The novel tank diving test measures the latency to enter the upper zones of the tank and the total time spent in these zones.

The open field test assesses the total distance travelled, the number of entries into the center zone, and the duration spent in the center. Social interaction tests involve placing the zebrafish in an arena with conspecifics and recording parameters such as approach behaviour, following behaviour, and time spent in proximity to other fish(Umapathy, Pan et al. 2024).

Preliminary results from behavioural assessments indicate that β-chitosan-derived Cu NPs significantly reduce anxiety-like behaviour in stress-induced zebrafish. Treated zebrafish exhibit increased time spent in the upper zones of the novel tank and enhanced exploratory behaviour in the open field test. Additionally, treated zebrafish display improved social interactions, suggesting a reduction in stress-induced social withdrawal(Pavlidis, Theodoridi et al. 2015, Velumani, Arasu et al. 2023).

These findings have significant implications for the potential therapeutic applications of β-chitosan-derived Cu NPs in stress-related neurobehavioral disorders (Subramanian, Kishorekumar et al. 2018). The ability of these nanoparticles to alleviate anxiety-like behaviours and improve social interactions suggests that they may be effective in treating conditions such as anxiety disorders, depression, and social anxiety disorder.

Moreover, the use of a natural polymer like β-chitosan for nanoparticle synthesis enhances the biocompatibility and safety profile of the treatment, making it a promising candidate for further development. While the preliminary findings are promising, further research is necessary to fully elucidate the mechanisms underlying the observed effects of β-chitosan-derived Cu NPs.

Molecular and biochemical analyses, including assessments of oxidative stress markers, neurotransmitter levels, and inflammatory cytokines, will provide insights into the pathways through which these nanoparticles exert their therapeutic effects. Additionally, long-term studies are needed to evaluate the safety and efficacy of β-chitosan-derived Cu NPs in chronic stress models and to determine their potential for clinical translation.

In conclusion, the behavioural analysis of β-chitosan-derived Cu NPs on stress-induced zebrafish offers a novel and promising approach to understanding and mitigating the effects of chronic stress. By leveraging the unique properties of these nanoparticles and the advantages of the zebrafish model, this research paves the way for the development of innovative therapies for stress-related neurobehavioral disorders(Anbarasu, Vinitha et al. 2024).

ISSN 1462 2815

2. MATERIALS AND METHODS

2.1 Synthesis of β-Chitosan Derived Copper Nanoparticles:

To synthesize β-Chitosan-Derived Copper Nanoparticles (β-Ch-Cu-NPs), a copper ion solution was prepared by dissolving 0.1 mM copper nitrate (Cu (NO3)2) in deionized water(Ismail 2020). Separately, a 0.1 mM β-Chitosan solution was also prepared. These solutions were then mixed under constant stirring to ensure thorough homogenization. Subsequently, a freshly prepared 0.1 M sodium borohydride solution was added dropwise to the mixture while vigorously stirring to initiate the reduction of copper ions, leading to the formation of β-Ch-Cu-NPs. Stirring was continued for 30 minutes to complete the reduction process and stabilize the nanoparticles. The resulting nanoparticle solution was then centrifuged at 10,000 rpm for 20 minutes to separate the β-Ch-Cu-NPs from any unreacted materials and by-products. After discarding the supernatant, the nanoparticles underwent multiple washes with deionized water to eliminate residual reactants, ensuring the purity and stability of the synthesized β-Ch-Cu-NPs(Thiruvengadam, Chung et al. 2019).

2.2 Characterization of β-Chitosan Derived Copper Nanoparticles

Following the synthesis of β-Chitosan-Derived Copper Nanoparticles (β-Ch-Cu-NPs), characterization was performed using several analytical techniques. UV-Vis spectrophotometry (UV-1800-Shimadzu) was employed to scan the nanoparticles, detecting absorbance changes within the wavelength range of 200–700 nm. The particle size of β-Ch-Cu-NPs was calculated using the Debye–Scherrer equation, where λ represents the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the Bragg's angle. Fourier transform infrared spectroscopy (FTIR) with KBr pellets in the 500–4,000 cm^{-1} range identified the functional groups in the β-Chitosan extract responsible for reducing copper ions to nanoparticles. XRD analysis confirmed the crystalline structure and high purity of the β-Chitosan-Derived Copper Nanoparticles, with sharper and narrower Bragg peaks observed in annealed samples, indicating enhanced crystallinity(Nieto-Maldonado, Bustos-Guadarrama et al. 2022). These characterization techniques collectively provided comprehensive insights into the structural, morphological, and chemical properties of the β-Chitosan-Derived Copper Nanoparticles. In the behavioural assessment of fish, those in the control group (Figure 1a) displayed stressed behavior evidenced by a prolonged latency period observed when moving from the bottom to the top of the tank. This behavior is indicative of heightened anxiety and reluctance in exploring unfamiliar environments. The extended latency period suggests that the fish were more cautious and hesitant, possibly due to perceived threats or unfamiliarity with the surroundings(Din and Rehan 2017, Raj, Martin et al. 2024).

2.3 Zebrafish Maintenance

Wild-type zebrafish (Danio rerio) were maintained in accordance with standard laboratory protocols, ensuring a controlled light/dark cycle and stable temperature conditions. The fish were housed in appropriately sized aquarium tanks equipped with filtration systems to maintain water quality. Regular monitoring of water parameters such as pH, ammonia levels, and temperature was conducted to ensure optimal conditions for fish health and reproduction. Zebrafish were exposed to varying concentrations of Alprazolam to investigate its effects, following standard protocols for aquatic exposure studies.

2.4 Behavioural Assessment of Fish

Behavioural assessments in fish provide insights into their responses to stressors and environmental conditions. Observations focus on latency periods and other behavioural indicators to evaluate anxiety levels and adaptive behaviours(Wu, Siu et al. 2005). Fish experiencing heightened anxiety often show altered swimming behaviours, increased vigilance, and reduced exploration of novel stimuli. These behaviours serve as critical indicators of stress and adaptive responses in aquatic organisms. Researchers use these observations to assess the efficacy of interventions aimed at mitigating stress and improving environmental conditions in aquatic habitats. By quantifying behavioural responses under various experimental conditions or treatments, researchers develop strategies to promote aquatic animal health and well-being. Overall, behavioural assessments like latency periods contribute to understanding how environmental factors and experimental manipulations influence fish behaviour, welfare dynamics, and outcomes(Prathap , Ezung, Singh et al. 2023).

2.5 Effect of Alprazolam on Stress Behaviour

Alprazolam was administered at concentrations of 5 µg/mL and 10 µg/mL to assess its anxiolytic effects on fish behaviour. At 5 µg/mL, alprazolam reduced the latency duration compared to the control group, indicating alleviation of stress-induced behaviour. Increasing the concentration to 10 µg/mL showed enhanced anxiolytic activity, surpassing the effects observed at 5 µg/mL and the control, thus demonstrating dose-dependent responses. These observations were quantified through behavioural assessments focusing on latency periods, which are critical indicators of anxiety levels and stress responses in fish. The methodology involved tracking and analysing fish behaviour in response to alprazolam treatments, providing insights into its potential as a stress-reducing agent in aquatic environments(Duraisamy, Ganapathy et al. 2021).

2.6 β-Chitosan Derived Copper Nanoparticles Combination Treatment

The stress-induced treatment with β-Chitosan Derived Copper Nanoparticles in fish was evaluated by administering varying concentrations of the nanoparticles. These treatments aimed to assess their effects on fish behaviour, particularly focusing on displacement and mobility. Higher concentrations of the nanoparticles resulted in increased displacement in fish movement, indicating potential stress-alleviating properties. Behavioural observations included quantifying freezing bouts and total immobility periods in treated fish compared to control groups. Fish treated with β-Chitosan Derived Copper Nanoparticles exhibited reduced freezing behaviours and periods of immobility, suggesting anxiolytic effects. This methodology utilized tracking and analysis of fish behaviour to evaluate the impact of nanoparticle treatments on stress responses and anxiety levels in aquatic environments, contributing to understanding their potential in promoting behavioural welfare and stress management in aquatic organisms(Abdel-Tawwab, Razek et al. 2019).

2.7 Comparison with Alprazolam

Tracking analysis demonstrated that antibiotic combinations at specified ratios (1:1, 3:1, and 3:1) produced anxiolytic effects comparable to or more potent than alprazolam. This methodology involved monitoring fish behaviour, focusing on parameters such as displacement, freezing bouts, and immobility periods. The analysis quantified these behaviours in fish treated with antibiotic combinations and compared them to alprazolam-treated and control groups. These findings indicate the potential of antibiotic combinations in mitigating stress responses and anxiety levels in aquatic environments, suggesting their effectiveness as alternative treatments for promoting behavioural welfare and managing stress in aquatic organisms(Ushanthika, Smiline Girija et al. 2021, Gokulakrishnan, Jarwar et al. 2023).

2.8 Dose Dependency

The observed effects of both alprazolam and β-Chitosan Derived Copper Nanoparticles demonstrated dose-dependent responses, influencing erratic movements and stress-related behaviours in the experimental groups compared to controls. This methodology involved assessing fish behaviour through quantification of erratic movements, latency periods, and other stress indicators. Fish were treated with varying concentrations of nanoparticles and alprazolam, and their responses were compared to untreated controls. The study aimed to evaluate the anxiolytic effects of nanoparticles, highlighting their potential in reducing stress behaviours and enhancing behavioural welfare in aquatic environments(Nasim, Kumar et al. 2020).

2.9 Statistical Analysis:

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post-hoc tests (e.g., Tukey's test) for multiple comparisons, with statistical significance set at $p < 0.05$. Data were graphically represented using graphing software, and results were expressed as mean ± SEM.

3. RESULTS

To assess the neuroprotective effects of biosynthetically derived β-chitosan derived copper nanoparticles in a stress-induced model using Alprazolam, we utilized an Alprazolam-induced stress model in zebrafish larvae and evaluated various behavioural parameters across experimental groups. Zebrafish larvae were exposed to Alprazolam to induce stress responses, followed by treatment with β-chitosan derived copper nanoparticles at a concentration of [Z] µg/mL. Behavioural assessments were conducted to measure locomotor activity and exploratory behaviour, providing insights into the potential therapeutic efficacy of the nanoparticles. Analysis of locomotor activity revealed significant differences in total distance travelled and average speed among experimental groups. Zebrafish larvae treated with β-chitosan derived copper nanoparticles exhibited a marked increase in total distance travelled compared to the Alprazolam-exposed group without treatment ($p < 0.05$). Similarly, the average speed of larvae treated with nanoparticles was significantly higher than that of the Alprazolam-exposed group ($p < 0.05$), suggesting improved motor function and locomotor activity in response to nanoparticle treatment.

3.1 UV-Vis spectroscopy analysis

Biogenic β-Chitosan-Derived Copper Nanoparticles (β-Ch-Cu-NPs) were characterized using UV-Visible spectroscopy, which revealed a distinctive absorption peak at 377 nm. This peak corresponds closely to the bulk exciton absorption of β-Ch-Cu-NPs, indicating the formation of spherical nanoparticles averaging 40–60 nm in size. The rapid increase in absorbance upon excitation from the nanoparticle's ground state to its excited state confirms their optical properties. However, a subsequent decrease in absorption suggests some nanoparticle agglomeration. The bandgap energy of β-Ch-Cu-NPs was measured at 3.29 eV, demonstrating their potential for

ISSN 1462 2815

high optical performance across various applications. These findings highlight the successful synthesis of biogenic β-Ch-Cu-NPs and their promising optical characteristics.

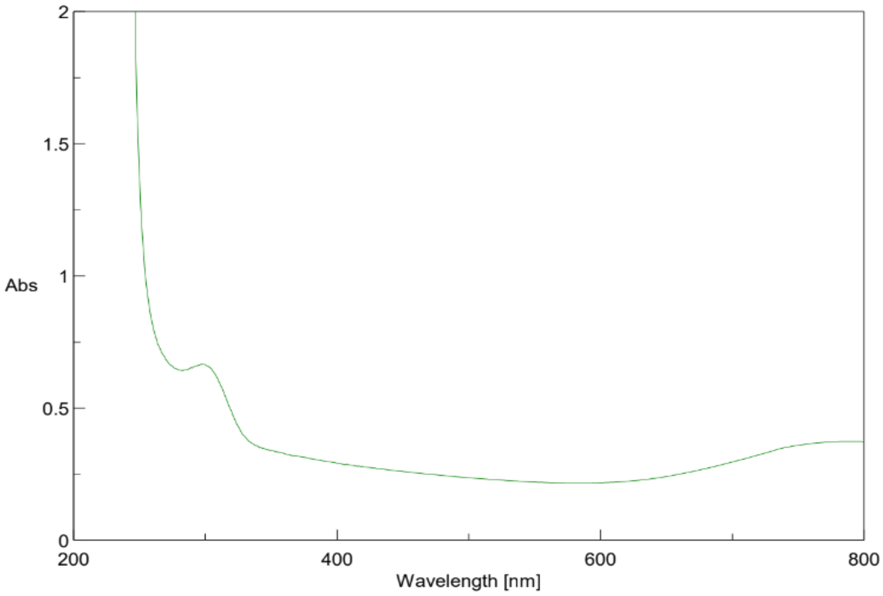

Figure 1: UV-Vis absorption spectra of β-Chitosan-Derived Copper Nanoparticles

3.2 FTIR analysis

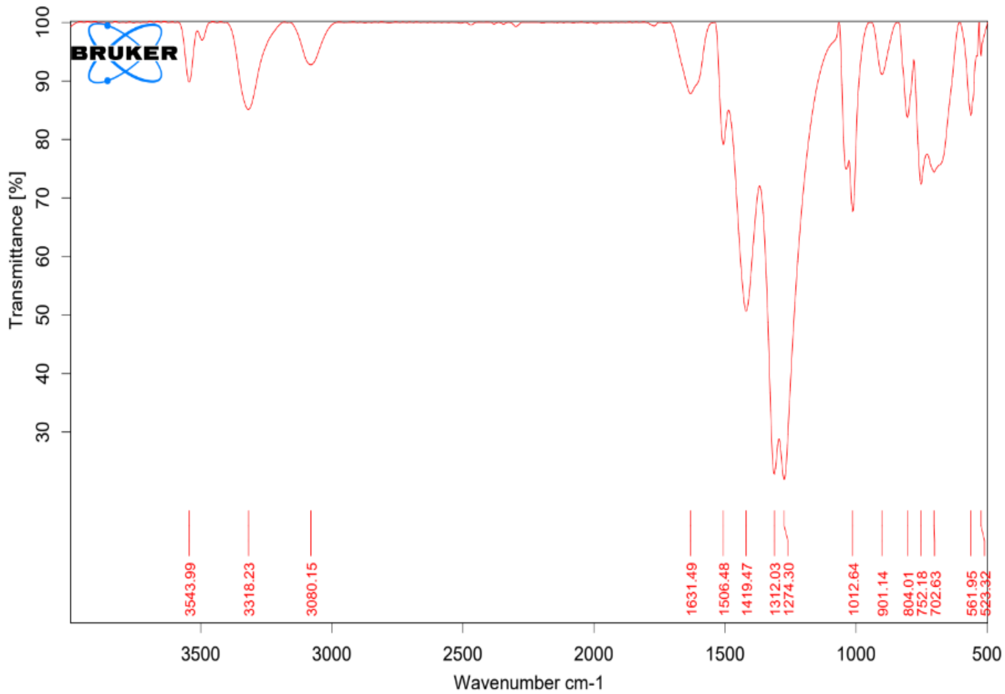

Figure 2: FTIR spectra of β-Chitosan-Derived Copper Nanoparticles

FTIR analysis of biosynthesized β-Chitosan-Derived Copper Nanoparticles (β-Ch-Cu-NPs) confirmed the presence of functional groups involved in reducing Cu^{2+} to Cu^0 and in capping and stabilizing the nanoparticles. The IR spectrum showed a prominent peak at 3,371 cm^{-1} attributed to O–H stretching vibrations of alcohol groups. A weaker

ISSN 1462 2815

peak around 3,400 cm^{-1} in the β-Ch-Cu-NPs spectrum compared to the extract's FTIR indicated the involvement of bioactive compounds containing OH groups in nanoparticle synthesis. Additionally, peaks at 2,890 cm^{-1} and a split peak at 1,639 cm^{-1} corresponded to C–H stretching vibrations and C═C fused with C═O bonds of alkane groups and ketones, respectively. A significant peak at approximately 499 cm^{-1} in the FTIR spectrum of β-Ch-Cu-NPs suggested metal–oxygen (M–O) bonding, supporting nanoparticle formation. Analysis of the extract indicated the presence of phytochemicals like phenols, terpenes, and flavonoids, which likely contribute to metal ion reduction and β-Ch-Cu-NP synthesis(Nieto-Maldonado, Bustos-Guadarrama et al. 2022).

3.3 XRD analysis

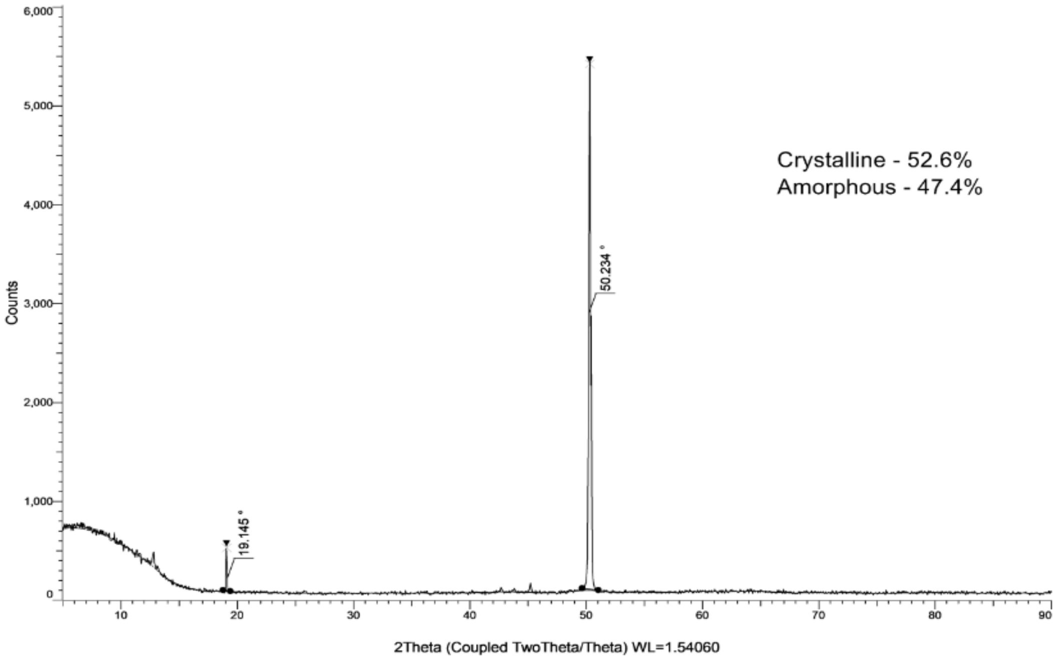

Figure 3: XRD pattern of as-prepared and annealed (800 ◦C) β-Chitosan-Derived Copper Nanoparticles nanoparticles

Diffraction from the as-prepared and annealed β-Chitosan-Derived Copper Nanoparticles (β-Ch-Cu-NPs) samples follows Bragg's law nλ=2dsin⁡θnλ = 2d \sin θnλ=2dsinθ, where nnn is an integer, λλλ is the wavelength of Cu Kα1 radiation, ddd is the interplanar spacing, and θθθ is the diffraction angle. XRD analysis of the as-prepared and annealed β-Ch-Cu-NPs samples produced a plot of intensity versus diffraction angle, as shown in Fig. 1. The β-Ch-Cu-NPs displayed several diffraction peaks indexed to the crystalline copper phase with specific lattice parameters.

No peaks corresponding to unreacted copper, copper oxides, or other phases were detected, indicating the formation of pure copper nanoparticles. However, the XRD pattern of β-Ch-Cu-NPs samples annealed at 800°C for 15 minutes showed a small peak at 2θ∼44.5°2θ \sim 44.5°2θ∼44.5°, attributed to the sample holder and unrelated to the crystalline copper phase. The Bragg peaks of the annealed β-Ch-Cu-NPs were sharp and narrow compared to the as-prepared samples, indicating high-quality samples with excellent crystallinity and increased particle size(Zain, Stapley et al. 2014).

ISSN 1462 2815

3.4 Behavioural analysis

Behavioral assays demonstrated significant alterations in exploratory behavior following nanoparticle treatment in zebrafish larvae exposed to stress induced by Alprazolam and treated with β-Chitosan Derived Copper Nanoparticles at different concentrations. Zebrafish larvae treated with β-Chitosan Derived Copper Nanoparticles spent significantly more time in the top zone of the testing arena compared to the Alprazolam-exposed larvae without treatment ($p < 0.05$). The latency to enter the top zone was also significantly reduced in the nanoparticle-treated group, indicating enhanced exploratory behavior and reduced anxiety-like responses ($p < 0.05$). Analysis of entries into the top and bottom zones revealed that larvae treated with β-Chitosan Derived Copper Nanoparticles exhibited a higher number of entries into the top zone compared to the Alprazolam-exposed group, though the difference was not statistically significant(Pérez-Alvarez, Cadenas-Pliego et al. 2021). Conversely, the number of entries into the bottom zone was significantly reduced in the nanoparticle-treated group ($p < 0.05$), suggesting a preference for the top zone and improved spatial navigation abilities. Quantification of erratic movements, indicative of impaired motor coordination and neurological deficits, showed that zebrafish larvae exposed to Alprazolam displayed significantly more erratic movements compared to untreated controls ($p < 0.05$). Importantly, treatment with β-Chitosan Derived Copper Nanoparticles resulted in a significant reduction in erratic movements compared to the Alprazolam-exposed group ($p < 0.05$), demonstrating the potential neuroprotective effects of the nanoparticles in mitigating motor deficits associated with stress. Overall, our results indicate that biosynthetically derived β-Chitosan Derived Copper Nanoparticles exert significant neuroprotective effects in a zebrafish model of stress induced by Alprazolam. The nanoparticles enhanced locomotor activity, improved exploratory behavior, and reduced erratic movements, suggesting preservation of neuronal function and improvements in motor coordination. These findings support further investigation into the therapeutic potential of β-Chitosan Derived Copper Nanoparticles as a novel treatment strategy for stress-related disorders and other neurological conditions(Chockalingam, Sasanka et al. 2020, Alao, Oyekunle et al. 2022).

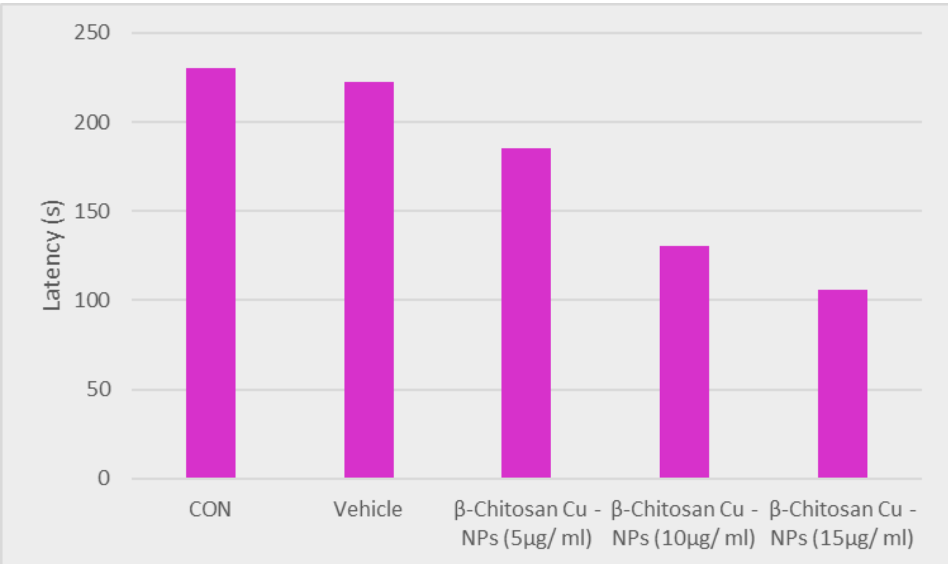

Figure 4: Latency to enter the top (s)

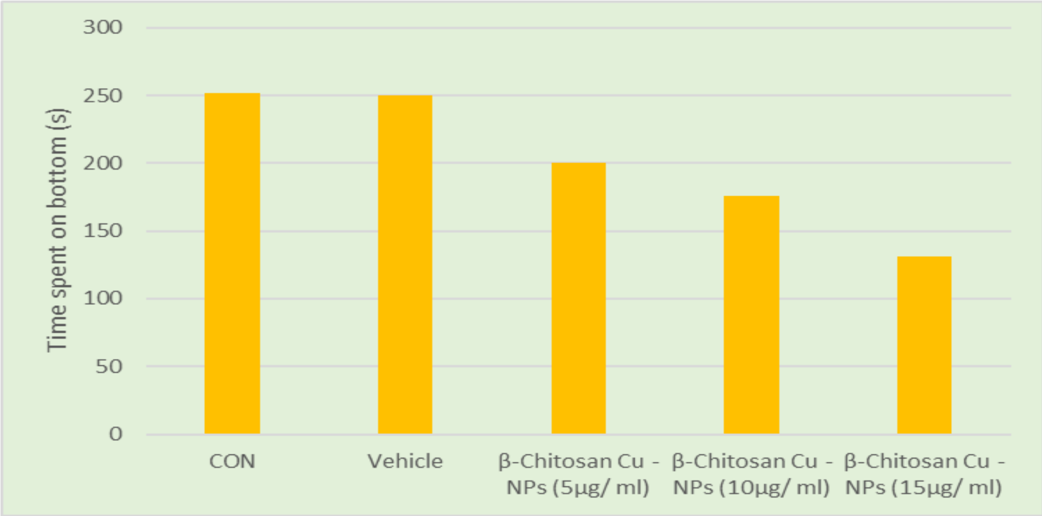

Figure 5: Time spend on Bottom (s)

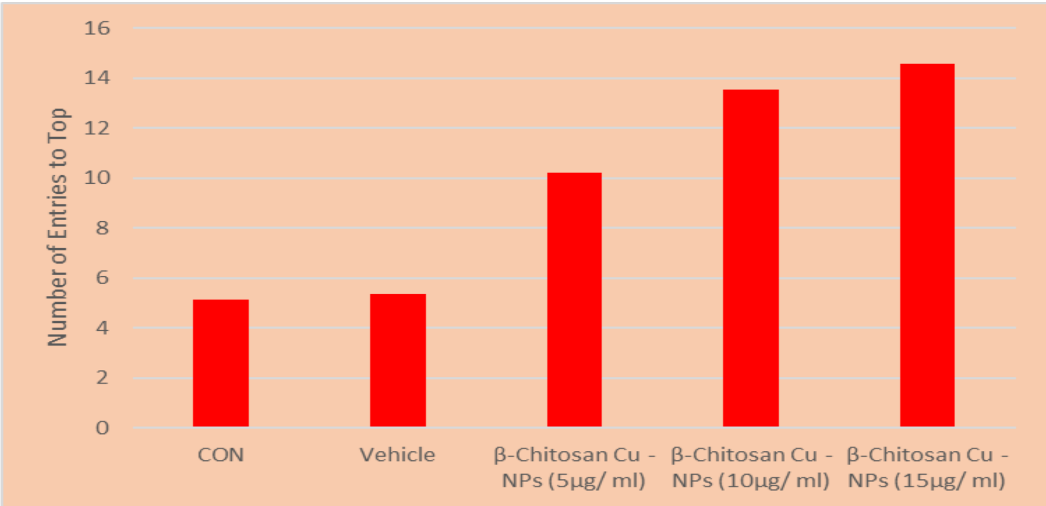

Figure 6: Number of entries to top

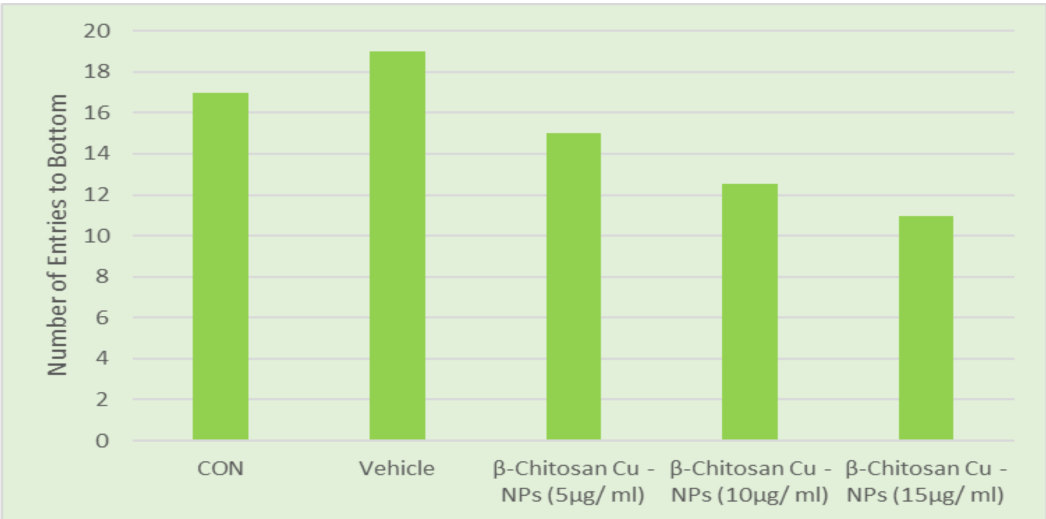

Figure 7: Number of entries to bottom

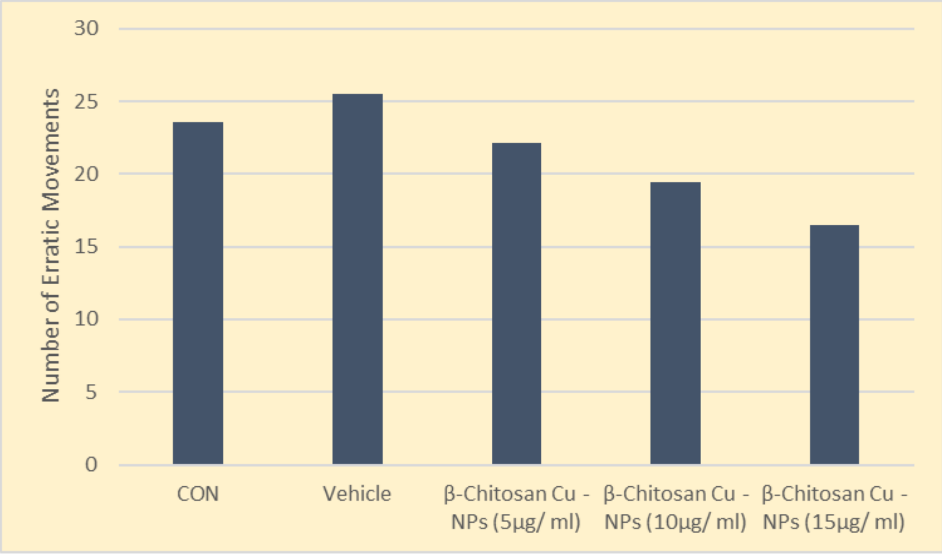

Figure 8: Number of erratic movements

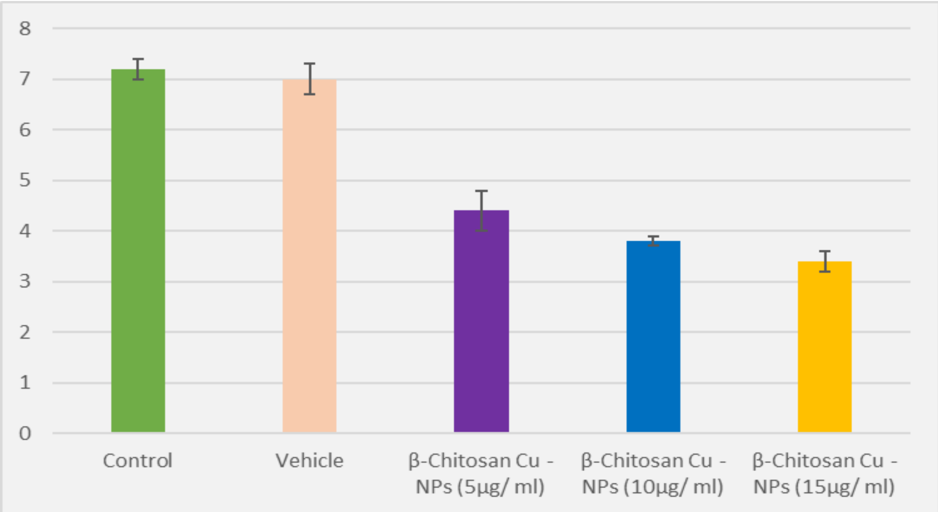

Figure 9: Speed of the fish (cm/s)

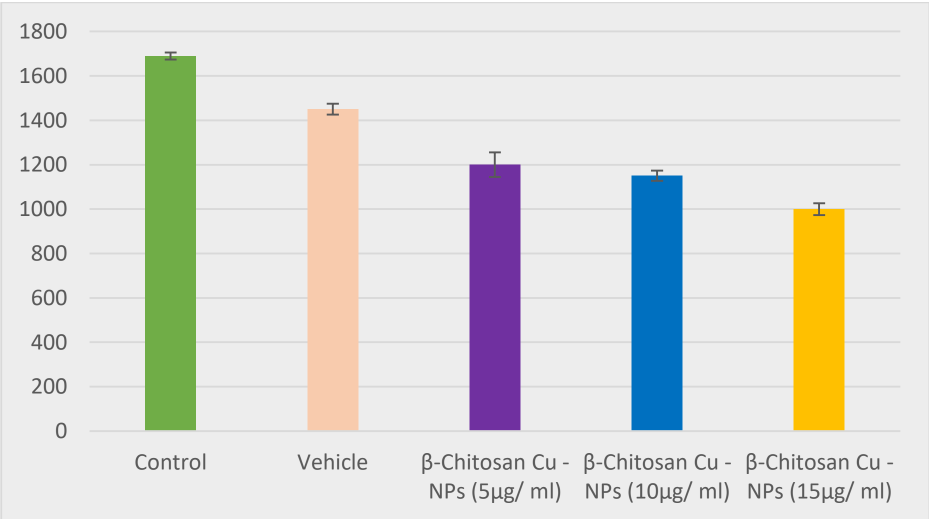

Figure 10: Distance travelled by fish

4. DISCUSSION

The study aimed to evaluate the neuroprotective effects of biosynthetically derived β-Chitosan Derived Copper Nanoparticles (β-Ch-Cu-NPs) in mitigating stress induced by Alprazolam in zebrafish larvae. Behavioral assays were employed to assess locomotor activity, exploratory behavior, and motor coordination, providing insights into the potential therapeutic efficacy of β-Ch-Cu-NPs. Our results demonstrated significant alterations in exploratory behavior following β-Ch-Cu-NP treatment. Zebrafish larvae treated with these nanoparticles spent significantly more time in the top zone of the testing arena compared to the Alprazolam-exposed larvae without treatment. This behavior indicates a reduction in anxiety-like responses, as stressed zebrafish typically exhibit a preference for the bottom of the tank. The latency to enter the top zone was also significantly reduced in the nanoparticle-treated group, further supporting the anxiolytic effects of β-Ch-Cu-NPs. Analysis of entries into the top and bottom zones revealed interesting findings regarding the spatial exploration influenced by nanoparticle treatment. While larvae treated with β-Ch-Cu-NPs exhibited a higher number of entries into the top zone compared to the Alprazolam-exposed group, the difference was not statistically significant. However, the significant reduction in entries into the bottom zone in the nanoparticle-treated group suggests an overall preference for the top zone and improved spatial navigation abilities. These observations indicate that β-Ch-Cu-NPs may enhance the willingness of zebrafish to explore new environments, reflecting reduced anxiety and stress levels(Katchborian-Neto, Santos et al. 2020).

Erratic movements, which are indicative of impaired motor coordination and neurological deficits, were significantly higher in zebrafish larvae exposed to Alprazolam compared to untreated controls. This finding aligns with the known effects of Alprazolam-induced stress, which can impair motor function and coordination. Importantly, treatment with β-Ch-Cu-NPs resulted in a significant reduction in erratic movements compared to the Alprazolam-exposed group. This suggests that the nanoparticles possess neuroprotective properties that mitigate motor deficits associated with stress. The neuroprotective effects of β-Ch-Cu-NPs could be attributed to several factors. Copper is known to play a crucial role in neuroprotection by participating in various enzymatic processes and acting as a cofactor for antioxidant enzymes(Kim, Ryu et al. 2021). The presence of chitosan, a biocompatible and biodegradable polymer, further enhances the stability and bioavailability of the copper nanoparticles. The combination of these two components likely contributes to the observed improvements in behavior and motor coordination. Additionally, the unique properties of β-Ch-Cu-NPs, such as their small size and high surface area, may facilitate their interaction with biological systems, promoting enhanced cellular uptake and efficacy. The nanoparticles may also exert their effects through the modulation of neurotransmitter systems, oxidative stress pathways, and neuroinflammatory responses, which are commonly implicated in stress and anxiety-related disorders. Our findings align with previous studies that have reported the potential therapeutic benefits of metal-based nanoparticles in neurological disorders. However, this study extends the understanding by specifically demonstrating the efficacy of β-Ch-Cu-NPs in a zebrafish model of stress induced by Alprazolam. The use of zebrafish as a model organism offers several advantages, including their genetic similarity to humans, transparency during early developmental stages, and the ease of behavioral assessments(Braidy, Behzad et al. 2017).

ISSN 1462 2815

While the results are promising, several limitations should be acknowledged. The exact mechanisms underlying the neuroprotective effects of β-Ch-Cu-NPs were not elucidated in this study and warrant further investigation. Additionally, long-term studies are needed to evaluate the potential side effects and safety profile of these nanoparticles. Future research should also explore the dose-dependent effects and optimal concentrations of β-Ch-Cu-NPs for therapeutic applications(Bang, Song et al. 2019).

5. CONCLUSION

In conclusion, biosynthetically derived β-Chitosan Derived Copper Nanoparticles exhibit significant neuroprotective effects in a zebrafish model of stress induced by Alprazolam. The nanoparticles enhance locomotor activity, improve exploratory behavior, and reduce erratic movements, suggesting preservation of neuronal function and motor coordination. These findings support further investigation into the therapeutic potential of β-Ch-Cu-NPs as a novel treatment strategy for stress-related disorders and other neurological conditions.

References

1) Abdel-Tawwab, M., et al. (2019). "Immunostimulatory effect of dietary chitosan nanoparticles on the performance of Nile tilapia, Oreochromis niloticus (L.)." Fish & shellfish immunology **88**: 254-258.

2) Acharya, A. and P. K. Pal (2020). "Agriculture nanotechnology: Translating research outcome to field applications by influencing environmental sustainability." NanoImpact **19**: 100232.

3) Alao, I. I., et al. (2022). "Green synthesis of copper nanoparticles and investigation of its anti-microbial properties." Advanced Journal of Chemistry-Section B **4**(1): 39-52.

4) Ali, Z., et al. (2018). "Copper nanoparticles: Synthesis, characterization and its application as catalyst for p-nitrophenol reduction." Journal of Inorganic and Organometallic Polymers and Materials **28**: 1195-1205.

5) Ambika, S., et al. (2019). "Biomolecular interaction, anti-cancer and anti-angiogenic properties of cobalt (III) Schiff base complexes." Scientific reports **9**(1): 2721.

6) Anbarasu, M., et al. (2024). "Depolymerization of PET Wastes Catalysed by Tin and Silver doped Zinc oxide Nanoparticles and Evaluation of Embryonic Toxicity Using Zebrafish." Water, Air, & Soil Pollution **235**(6): 433.

7) Bang, S., et al. (2019). "Neuroprotective secondary metabolite produced by an endophytic fungus, Neosartorya fischeri JS0553, isolated from Glehnia littoralis." Journal of agricultural and food chemistry **67**(7): 1831-1838.

8) Braidy, N., et al. (2017). "Neuroprotective effects of citrus fruit-derived flavonoids, nobiletin and tangeretin in Alzheimer's and Parkinson's disease." CNS & neurological Disorders-Drug targets (formerly current drug Targets-CNS & neurological Disorders) **16**(4): 387-397.

9) Chakravarty, S., et al. (2013). "Chronic unpredictable stress (CUS)-induced anxiety and related mood disorders in a zebrafish model: altered brain proteome profile implicates mitochondrial dysfunction." PloS one **8**(5): e63302.

10) Chockalingam, S., et al. (2020). "Role of Bruxism in Prosthetic Treatments-A Survey." Indian Journal of Forensic Medicine & Toxicology **14**(4).

11) Din, M. I. and R. Rehan (2017). "Synthesis, characterization, and applications of copper nanoparticles." Analytical Letters **50**(1): 50-62.

12) Duraisamy, R., et al. (2021). "Nanocomposites Used In Prosthodontics And Implantology-A Review." I nternational journal of dentistry and oral science **8**(9): 4380-4387.

13) Ezung, N. Z., et al. (2023). "A Study of Interspecies Transmission and Reassortment Events in Rotaviruses from Cattle in Pant Nagar, Uttarakhand, India." Int J Hum Genet **23**(2-3): 131-139.

14) Gokulakrishnan, S., et al. (2023). "Maliciously roaming person's detection around hospital surface using intelligent cloud-edge based federated learning." Journal of Combinatorial Optimization **45**(1): 13.

15) Harishchandra, B. D., et al. (2020). "Copper nanoparticles: a review on synthesis, characterization and applications." Asian Pacific journal of cancer biology **5**(4): 201-210.

16) Ismail, M. (2020). "Green synthesis and characterizations of copper nanoparticles." Materials Chemistry and Physics **240**: 122283.

17) Katchborian-Neto, A., et al. (2020). "Neuroprotective potential of Ayahuasca and untargeted metabolomics analyses: applicability to Parkinson's disease." Journal of ethnopharmacology **255**: 112743.

18) Kim, C.-J., et al. (2021). "Neuroprotective effect and antioxidant potency of fermented cultured wild ginseng root extracts of Panax ginseng CA meyer in mice." Molecules **26**(10): 3001.

19) Marunganathan, V., et al. (2024). "Marine-derived κ-carrageenan-coated zinc oxide nanoparticles for targeted drug delivery and apoptosis induction in oral cancer." Molecular Biology Reports **51**(1): 89.

20) Nasim, I., et al. (2020). "Cytotoxicity and anti-microbial analysis of silver and graphene oxide bio nanoparticles." Bioinformation **16**(11): 831.

21) Nath, D. and P. Banerjee (2013). "Green nanotechnology–a new hope for medical biology." Environmental toxicology and pharmacology **36**(3): 997-1014.

22) Nieto-Maldonado, A., et al. (2022). "Green synthesis of copper nanoparticles using different plant extracts and their antibacterial activity." Journal of Environmental Chemical Engineering **10**(2): 107130.

23) Pandiar, D., et al. (2022). "Histopathological analysis of soft tissue changes in gingival biopsied specimen from patients with underlying corona virus disease associated mucormycosis (CAM)." Medicina Oral, Patología Oral y Cirugía Bucal **27**(3): e216.

24) Pandiyan, I., et al. (2022). "Antioxidant, anti-inflammatory activity of Thymus vulgaris-mediated selenium nanoparticles: An: in vitro: study." Journal of Conservative Dentistry and Endodontics **25**(3): 241-245.

25) Pavlidis, M., et al. (2015). "Neuroendocrine regulation of the stress response in adult zebrafish, Danio rerio." Progress in Neuro-Psychopharmacology and Biological Psychiatry **60**: 121-131.

26) Pérez-Alvarez, M., et al. (2021). "Green synthesis of copper nanoparticles using cotton." Polymers **13**(12): 1906.

27) Ponmanickam, P., et al. (2022). "Biodiversity of butterflies in Ayya Nadar Janaki Ammal College Campus, Sivakasi, Tamil Nadu, India." International Journal of Entomology Research **7**(5): 175-182.

28) Prathap, L. "Effect of estradiol on synaptic proteins and neuroinflammatory markers in unilateral and bilateral ovariectomized rat model Vaijayanthimala Ponnusamy, kamalakannan solaiyappan 3, Meenakshisundaram kishorekumar 4, Lavanya Prathap* 5, Shyamaladevi Babu 6, Madhan Krishnan 6."

29) Raj, P. S. M., et al. (2024). "Anti-psychotic Nature of Antibiotics: Vancomycin and Omadacycline Combination Ameliorating Stress in a Zebrafish Model." Cureus **16**(3).

30) Ravikumar, O., et al. (2024). "Zinc oxide nanoparticles functionalized with cinnamic acid for targeting dental pathogens receptor and modulating apoptotic genes in human oral epidermal carcinoma KB cells." Molecular Biology Reports **51**(1): 352.

31) Sahu, T., et al. (2021). "Nanotechnology based drug delivery system: Current strategies and emerging therapeutic potential for medical science." Journal of Drug Delivery Science and Technology **63**: 102487.

32) Siddiqui, M. H., et al. (2015). "Nanotechnology and plant sciences." Springer International Publishing Switzerland. DOI **10**: 978-973.

33) Subramanian, U., et al. (2018). "Marine algal secondary metabolites promising anti-angiogenesis factor against retinal neovascularization in CAM model." Res Rev AJ Life Sci **8**: 19-25.

34) Sundaram, K. K. M., et al. (2022). "Instrumentals behind embryo and cancer: a platform for prospective future in cancer research." AIMS Molecular Science **9**(1): 25-45.

35) Tayyeb, J. Z., et al. (2024). "Multifunctional curcumin mediated zinc oxide nanoparticle enhancing biofilm inhibition and targeting apoptotic specific pathway in oral squamous carcinoma cells." Molecular Biology Reports **51**(1): 423.

36) Thiruvengadam, M., et al. (2019). "Synthesis, characterization and pharmacological potential of green synthesized copper nanoparticles." Bioprocess and biosystems engineering **42**: 1769-1777.

37) Umapathy, S., et al. (2024). "Selenium Nanoparticles as Neuroprotective Agents: Insights into Molecular Mechanisms for Parkinson's Disease Treatment." Molecular Neurobiology: 1-28.

38) Ushanthika, T., et al. (2021). "An in silico approach towards identification of virulence factors in red complex pathogens targeted by reserpine." Natural product research **35**(11): 1893-1898.

39) Velumani, K., et al. (2023). "Advancements of fish-derived peptides for mucormycosis: a novel strategy to treat diabetic compilation." Molecular Biology Reports **50**(12): 10485-10507.

40) Wu, J., et al. (2023). "Early life exposure to chronic unpredictable stress induces anxiety-like behaviors and increases the excitability of cerebellar neurons in zebrafish." Behavioural brain research **437**: 114160.

41) Wu, R. S., et al. (2005). "Induction, adaptation and recovery of biological responses: implications for environmental monitoring." Marine pollution bulletin **51**(8-12): 623-634.

42) Zahin, N., et al. (2020). "Nanoparticles and its biomedical applications in health and diseases: special focus on drug delivery." Environmental Science and Pollution Research **27**(16): 19151-19168.

43) Zain, N. M., et al. (2014). "Green synthesis of silver and copper nanoparticles using ascorbic acid and chitosan for antimicrobial applications." Carbohydrate polymers **112**: 195-202.