Characterization of Green Synthesis VA-Pd/AC NPs and Evaluation of Their Neurotoxicity Using the Zebrafish Larvae

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Abstract

New strategies for fast, efficient and environmentally friendly approaches in nanoparticle (NP) synthesis have increased the interest in less toxic green synthesis products. In this study, VA-Pd / AC nanoparticles (NPs) were synthesized using palladium (Pd), activated carbon (AC) and *Viscum album* (VA) aqueous extract as stabilizer / reductant. Activated carbon in the synthesis stage was obtained using the *Chenopodium album* (CA) plant. The structural and morphological properties of VA-Pd / AC NPs were examined using TEM and XRD analyses. Zebrafish embryo and larvae were exposed to the produced VA-Pd / AC NPs at different concentrations (10, 50, 100 and 500 mg/L) for 96 h. Embryos/larvae were examined for survival rate, hatching and malformation during development. Morever, potential neurotoxic effects were assessed histopathologically and immunohistochemically using Caspase 3 and nNOS indicators.

Although the hatching rate of VA-Pd / AC NP was 100% at all concentrations, the survival rate decreased in a dose dependent manner. Histopathological analyses revealed that 10 mg/L group looked similar to the control group, whereas necrotic degenerations were observed in the 50, 100 and 500 mg/L groups in a dose- dependent manner. In addition, Caspase 3 and nNOS expressions were evaluated as negative in the control and 10 mg/L groups in immunofluorescence staining.

These results provide the evidence that 500 mg/L concentration of green synthesized VA-Pd/AC NPs from Viscum album (VA) and Chenopodium album (CA) plant induces neurotoxic and tetragenonic effect on zebrafish.

Introduction

Recently, therapeutic agents include a wide range of materials, among which metal and metal oxide nanoparticles (NPs) have received great attention. The biosynthetic approach of these NPs is a convenient and superior alternative to the traditional physical and chemical methods used in NP production. These sustainable, efficient, environmentally friendly and low-cost techniques are scalable with desired control properties of NPs' particle size and morphology (Shi et al., 2017). In addition, these biological agents can be effective in reducing the toxicity that occurs in conventional procedures (Rambabu et al., 2021).

Among green application agents, platinum NPs (Pt NPs) and palladium NPs (Pd NPs) have attracted attention as promising tools in biomedical applications and treatment of certain diseases (Fahmy et al., 2021). Chemical approaches used for the production of Pd NPs rely on the use of harsh reagents and synthetic capping agents. Therefore, biological systems such as microorganisms, seaweed and plant extracts have been proposed for the simple, safe and cost-effective production of NPs. In addition to being easy to prepare, plant extracts are used as natural reducers to reduce palladium (II) ions by virtue of the phytochemicals they contain (Fahmy et al., 2021). Recently, some studies have reported the green synthesis of bimetallic NPs using plant extracts (Fahmy et al., 2020; Fahmy et al., 2021).

The biological syntheses of NPs is more advantageous than chemical and physical syntheses in that they are environmentally friendly, commercially viable and economical. Therefore, plant extracts are of great interest in the green synthesis of NPs. Plants provide positive manipulation and control over crystal growth and stabilization. In this way, the plant-mediated synthesis of silver NPs is highly effective in achieving the desired shape, size and variation. Plants are widely used for the synthesis of AgNPs due to their easy availability, low cost, important bioactive compounds, non-toxicity and environmental friendliness. The synthesis of AgNPs using aromatic and medicinal plants has revealed a wide range of biological activities that include anticancer, antioxidant, antibacterial, antiviral, antifungal and anti-inflammatory activities (Gecer et al., 2021).

Recently, plants are also used to reduce the cost of activated carbon (AC) production. AC is a versatile and excellent adsorbent and is widely used thanks to its large surface area, micro-porous structure, high pore volume and high surface reactivity. AC is used in treatment of industrial wastewater, adsorptive removal of color, odor and taste from drinking water and other unwanted organic and inorganic contaminants, air purification, purification of many chemical, food and pharmaceutical products, various gas phase applications, energy storage, electrochemical application and catalysis (Aslan, 2021). The leaves of the semi-parasitic plant *Viscum album* (VA), an important bioactive source of lectins, viscoxins, alkaloids, flavonoids, and phenolic acids, have been reported to inhibit cytotoxicity via immunostimulating, apoptotic and anti-cancer activities (Burdějová et al., 2021).

In this study, AC-Pd NPs and VA leaf extract were used for green synthesis of VA-Pd / AC NPs. With the investigation of NPs for their neurotoxic activities, a modeling study was carried out by trying to define survival, physical and neurological changes in possible action mechanisms.

2. Materials And Methods

2.1. Plant Sample

Chenopodium album (CA) were collected from the campus area of Iğdır University, Turkey, for thir research. *Viscum album* (VA) leaf was collected in Erzurum region, Turkey. $PdCl_2$ was supplied at Sigma Aldrich company. The chemicals were used directly without any purification.

2.2. Preparation and characterization of VA-Pd/AC NPs nanoparticles

a. Preparation of activated carbon

Chenopodium album (CA) is known as a highly abundant invasive plant that grows in and around Igdir University. It was evaluated as the pre-eminent material for the activated carbon source. After the CA was washed, it was preserved in an oven for 24 hours at 80 ° C. Then, it was ground in intervals of 150-250 micrometers. It was kept for 30 minutes (in nitrogen environment) at a constant speed of 5^oC/minute at 600 °C, for carbonization. The sample was cooled to room temperature. It was left in 0.1 M HCl solution

throughout the day. Then, it was filtered and washed with distilled water until pH 7. The sample was left to be preserved at 105 °C for 24 hours.

b. *Viscum album* extracts preparation

In this research, *Viscum album* (VA) extract was used as the reducing agent. The (VA) leaf was thoroughly washed three times with distilled water to remove impurities and allowed to dry for 24 hours at 105 ° C. The dried VA samples were ground into very small pieces. A solution, containing 100 mL of deionized water and 10 g of VA was prepared, for VA extracts. The resulting mixture of VA was boiled for 2 h, and cooled down to room temperature. Then it was made ready to work by being filtered under vacuum.

c. Synthesis of VA-Pd/AC NPs

In green synthesis of VA-Pd/AC NPs, a solution containing 0.02 g of PdCl2, 0.02 g of CA in 40 mL of deionized water and 10 mL of VA extract were prepared in a glass vial and vigorously mixed at room temperature. The progress of the reaction was controlled by the color change that shows nanoparticle forming (Leal-Duaso et al., 2021). Thereafter, this solution was centrifuged at 12000 rpm for 10 minutes. The obtained sample was washed thoroughly and kept in the oven at 75 ° C for 24 hours (Fig. 1).

d. Characterization

X-ray diffraction (XRD) analyzes of VA-Pd/AC NPs were performed with Panalytical EMPYREAN device (Malvern Panalytical Ltd, Malvern, United Kingdom) operating at 45 kV and capable of CuKα radiation. XRD analyzes were done to examine the XRD peaks and crystal size of VA-Pd/AC NPs. In addition, to reveal the distribution of Pd metal nanoparticles on AC surface and the average particle size, transmission electron microscopy (TEM) analyzes were performed with the Hitachi HT7800 (Hitachi Ltd, Tokyo, Japan) device operating at 200 kV. The average particle size was calculated using Imagej and origin package programs by counting spherical particles in TEM images (Calimli, 2020).

2.3. In vivo study

a. Zebrafish

As living material of the research, adult wild-type zebrafish were maintained from Izmir Biomedicine and Genome Center. The used zebrafish larvae were younger than 5-days-old. So no need to require any license (Directive 86/609/EEC and EU Directive, 2010/63/EU). They were kept in 28°C with a 14-h light/10-h dark cycle, according to standard procedures (Westerfield, 1995). Fish were fed twice a day. In the first meal at 9 a.m. flake food (TetraMin Tropical Flakes 48% protein, 8% fat, and 2% fiber) were used, and in the second one, at 3 p.m. feeding was done with artemia.

b. Zebrafish embryo Exposition to VA-Pd/AC NPs

The study was planned based as a semi-static test with 4 replications. For preparing the different trial concentrations (10, 50, 100 and 500 mg/L); VA-Pd/AC NPs (2000 mg/L) stock solution had sonicated

(50/60 kHz, Huber Minichiller Diagenode, Germany) in ultra-pure water with E3 (0.17 mM KCl, 0.33 mM $MgSO_4$, 5 mM NaCl, and 0.33 mM $CaCl_2$) for 3 h. These solutions were renewed daily and before solution regeneration, sonications were done for 30 min (Federici et al., 2007; Rocco et al., 2015). This procedure is known to be an effective method for dispersing and preventing agglomeration of nanoparticles of water suspensions (Sato et al., 2008).

The trial was organised of five groups. The first one was as control group (the embryo was preserved in E3 medium). For each group, 30 embryos were used and embryos were preserved in E3 medium. In the experiment, embryos were added to 5 ml test solution (VA-Pd/AC NPs) in different 5 plates each one including 30 embryos for 96 hpf. Each trial medium was renewed daily and all embryo groups were exposed to nanoparticle solution at the same conditions (28°C).

c. Determination of survival rate, hatching rate, and embryo morphological alterations

The larvae and embryos of all groups were visualized by a stereomicroscope (SZX16 Olympus microscope with SC50 Olympus camera) for evaluating the alterations after the application time (24-96 h). Not observe heartbeat in larvae at 24 hpf was accepted as not living.

2.4. Histopatological examination

The zebrafish larvae samples were detected in 4% paraformaldehyde solution for 48 hours. Following the routine tissue procedure, the larvae were embedded in paraffin blocks and 4 µm thick sections were taken from the blocks. Histopathological sections were stained with the hematoxylin-eosin (HE) staining procedure and examined with light microscopy (Olympus BX 51, Germany). Findings were evaluated as none (-), mild (+), moderate (++) and severe (+++) according to immune positivity.

2.5. Immunfloresans examination

After deparaffinization and dehydration processes, the sections (taken on poly-l-lysine slides) were kept in distilled water. It was boiled 2 times for 5 minutes to mask the antigens in the cells and allowed to cool at room temperature. Protein block was dropped onto the sections, washed with PBS for 10 minutes. Primary antibodies (caspase-3, Cat No: sc-56053 Diluent Ratio: 1/100, Santa Cruz, nNOS Cat No: ab16650 Diluent Ratio: 1/100, Abcam) were prepared and applied according to the usage conditions. After washing with PBS, an immunofluorescence antibody was used as a secondary marker (FITC Cat No: ab6717 Diluent Ratio: 1/1000, Abcam) and was washed in distilled water after being kept in the dark for 45 minutes. Then, DAPI with mounting medium (Cat no, D1306 diluent ratio: 1/200, Thermo) was dropped and kept in the dark for 5 minutes, and the tissues were washed with distilled water, covered with a coverslip. The sections were examined under a fluorescence attachment microscope (Zeiss Leica DM 1000 Germany). The findings were evaluated as none (-), mild (+), moderate (++) and severe (+++) according to immune positivity.

2.6. Statistical analyses

Statistical analysis of all data was performed by the SPSS program (SPSS for Windows, version 20.0). All data are given with statistical results, standard error, and mean values. The difference between the groups was determined by one-way (ANOVA) analysis of variance and then evaluated by Tukey's test ($p < 0.05$). The Kruskal-Wallis test (one of the nonparametric tests) was used for the analysis of the differences among the groups data (obtained semi-quantitatively) in histopathological examination, and the Mann Whitney U test was used for the comparison of paired groups.

3. Results

3.1. Characterization of VA-Pd/AC NPs by XRD and TEM

XRD analysis of VA-Pd/AC NPs is given in Figure 2a. The peaks detected at 26.92^{o} and 44.81^{o} are specific for the element carbon as described previously (Fernandez-Ruiz et al., 2021). Despite slight deviations, the peaks observed in the range of 37.89-87.19^{o} were very close to the surface-centered cubic Pd NP (Fernandez-Ruiz et al., 2021). This is likely caused by the addition of VA to the material structure. To examine the distribution of Pd nanometals on the AC surface TEM analyses were performed (Figure 2). Although metal nanoclusters were generally found to be distributed in a uniform manner, some of them were obtained in light clusters. The average size of spherical particles was calculated as 7.14 nm from TEM images (Figure 2c).

3.2. The effect of VA-Pd / AC NPs on the survival rate, hatching and morphology of the larvae

Survival rate of zebrafish embryos/larvae exposed to VA-Pd/AC NP for 96 h decreased in a dose-dependent manner. When the control and treatment groups were compared, a significant difference was detected in all groups (Fig 3, $p < 0.05$).

To determine their potential toxicity, zebrafish embryos were exposed to green synthesized VA-Pd/AC NPs at concentrations of 10, 50, 100 and 500 mg/L. At all concentrations of VA-Pd / AC NPs, 100% of larvae hatched at the end of the 96th hour. When the larval hatching rates were examined, no significant difference was observed between the control and treatment groups at the 72nd and 96th hours (Fig 4, p□0.05). However, at the 48th hour, hatching rates decreased to 40%, 20% and 10% in the 50, 100 and 500 mg/L treatment groups, respectively (Fig 4, $p<0.05$). While there were no detectable malformations in 10, 50 and 100 mg/L treatment groups, 10% of embryos exhibited yolk sac edema and spine curvature in 500 mg/L group (Fig 5).

3.3. Evaluation of the effect of VA-Pd / AC NPs Histopathological and Immunhistochemical Findings

While the control and 10 mg/L groups were found to have normal histological appearance, significant necrosis and degeneration in neurons was observed in the 50, 100 and 500 mg/L groups (Figure 6, p□0.05). Caspase 3 is the final enzyme involved in the apoptosis mechanism (Glushakova et al., 2018). In other words, after the cell enters this path, it cannot be returned and must undergo apoptosis. Because of this feature, liver, kidney and brain tissue appear as frequently used markers in experimental studies

(Onaolapo et al., 2019). The present study, Caspase 3 and nNOS expressions were evaluated as negative in the control and 10 mg/L groups. In the 50 mg/L group, cytoplasmic caspase 3 and nNOS expression was detected in neurons at mild levels. Expression of both markers increased to moderate and severe levels in the 100 mg/L and 500 mg/L groups, respectively (Figure 6).

4. Discussion

Green synthesis has emerged as an alternative to traditional synthesis methods in the field of nanomaterial production. Several studies has applied green synthesis in synthesis of NPs using metals or their oxidized forms (iron, tin, copper, zinc, palladium, manganese, silver and gold) and plant extracts (green tea, black tea, ginger, turmeric, pomegranate, lemon, rose water, spinach, clove, cinnamon) (Nartop 2019; Fahmy et al., 2020; Fahmy et al., 2021; Gecer et al., 2021; Panda et al., 2021). Phytochemicals including total phenolic acid, flavonoid, tannin, terpene, coumarin, lycopene, vitamins, carotenoids and anthocyanins contained in the leaves, stems or roots of plants have been used as reducing agents (Burdějová et al., 2021).

Green medium methods have been used to prepare small-sized and crystalline metal oxide NPs (Gholoobi, 2017). In this study, we produced the VA-Pd/AC NPs that have unique size- and shape-related properties so may have great interest to use as nanoparticles formed with common metals. Based on these results, various VA primary / secondary phytochemicals (flavonoids and phenolic acids) function as stabilizing and coating agents during AgNP production as in VA-Pd/AC NPs. Therefore, these extract phytochemicals can be effective in reducing Ag nanoparticles and their toxicity (Mehwish et al., 2021). In the present study, VA-Pd/AC NPs were obtained with an average size of 7.14 nm and a spherical shape. This size is considerably smaller than that of the previously reported green synthesized NPs (10.2 nm and 18.12 nm) prepared using leaf extract (Raj et al., 2018; Amer and Awwad, 2021; Mehwish et al., 2021).This difference is likely due to use of different carriers, activated carbon source and/or the method and the plant bioactive components. Thus, the solvent used for the extraction of AV leaves is considered to affect the success of Pd NP synthesis and also control NP size and distribution (Panda et al., 2021).

Despite their small size, VA-Pd /AC NPs exerted low toxicity likely due to the fact that VA has anticancer, antimycobacterial, antiviral, apoptosis-inducing and immunomodulatory activities.VA extracts have also been shown to reduce the harmful and mutagenic effects of oxygen-free radicals generated (Önay Ucar et al., 2006).

The early stage of embryos is the most sensitive stage to external factors such as toxic chemicals and mechanical stress (Anila et al., 2021a). Our results had shown that high doses of Pd NPs interfere with normal hatching rate and survival and cause teratological abnormalities in the zebrafish larvae. Consistent with our results, Alafiatayo et al (2019) reported embryotoxic and teratogenic changes (egg coagulation, hatching, developmental deformities in somites, tail detachment, otolith, blood circulation, heartbeat, motility and skeletal malformation) on zebrafish development.

Parallel to our results, Kumari et al. (2017) reported that the NPs synthesized with *Calotropis gigantea* caused abnormalities such as yolk sac edema and curved body axis in zebrafish embryos and larvae. It is possible that chorions greatly protect embryos by slowing the diffusion of extracts to embryos and hence delays the effect of toxicants until hatching. Delayed hatching could also be due to the absence of hatching enzymes such as the zebrafish hatchingenzyme 1 (ZHE1), secreted by zebrafish hatching glands (İsmail et al., 2017). Evidence of the effect of compounds that cause hatching gland dysfunction is well documented during exposure to NPs and methyl mercury (İsmail et al., 2017).

Nitric oxide (NO) synthesis is catalyzed by nitric oxide synthase (NOS). There are three types of NOS: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). nNOS has been reported to prevent brain damage as well as physiological functions such as learning, memory and neurogenesis (Förstermann and Sessa, 2012; Zhang et al., 2018). In other words, it had been determined that it functions as an important defense mechanism in the brain tissue (Zhang et al., 2018).

Apoptosis is typically induced by the death receptors (extrinsic pathway) or the mitochondria (internal pathway). In both pathways, active caspase 3 and 7 cleave poly-ADP-ribose polymerase 1 (PARP1) in response to DNA damage (Artun and Karagöz, 2021). In this study, the plant extract's toxic effect caused damage in the brain due to the dose increase and obtained with histopathological and immunofluorescence staining. In low dose applications of *V. album* lectins (used in the synthesis of VA-Pd / AC NPs) are thought to regulate the genes involved in these processes. This situation is thought to be due to the prevention of migration and invasion here (Menke et al., 2021). In high dose applications, VA-Pd / AC NPs caused an increase in caspase 3 activity and caspase dependent cell death was induced. Some secondary metabolites found in plant extracts may be effective in these changes (Hosami et al., 2021). A previous study demonstrated the therapeutic effects of these plant components (Burdějová et al., 2021). With the present study, it was determined that *V album* affects the expression of Caspase 3 with other compounds such as flavonoids it contains. Similar mechanism has been explained by Kusmardi et al (2021) and it has been stated that flavonoid species are effective in the expression of apoptotic markers such as Caspase-3 (caspase-independent). In our research findings, necrosis data were found to be compatible with caspase expression. Necrosis, as a known condition, can occur at the same time with apoptosis (Artun and Karagöz, 2021).

In the present study, it was observed that necrotic degenerative alterations occurred depending on the dose increase. Similar to our research findings, Anila et al. (2021a) reported that palladium nanoparticles induced histopathological lesions in the adult zebrafish brain. It had also been found that green synthesis palladium nanoparticles cause abnormal histopathological changes in the liver tissues of adult zebrafish (Anial et al., 2021b)

Conclusion

VA-Pd /AC NPs were synthesized with high stability - environmental friendly method and their toxicological effects on zebra embryo/larvae were revealed. These results provide the evidence that VA-Pd/AC NPs have low toxicological effects. Hovewer high concentration VA-Pd /AC NPs induces apoptotic death via activation of caspase cascades. It has been revealed that VA-Pd / AC NPs have low particle size by characterization studies. Therefore, it holds promise in drug delivery as well as in its potential for therapeutic effects.

In this study, positive effects of VA-Pd / AC NPs (prepared with VA extract) on survival rate, hatching, and malformation as well as Caspase 3 and nNOS activity were supported by histopathological findings. However, more research is needed to elucidate in detail the underlying molecular action mechanism and the exact role of each derivative of these plant extracts. Therefore, it is highly recommended to break down the plant sap and investigate the affected molecular pathways to purify the active compounds.

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Figures

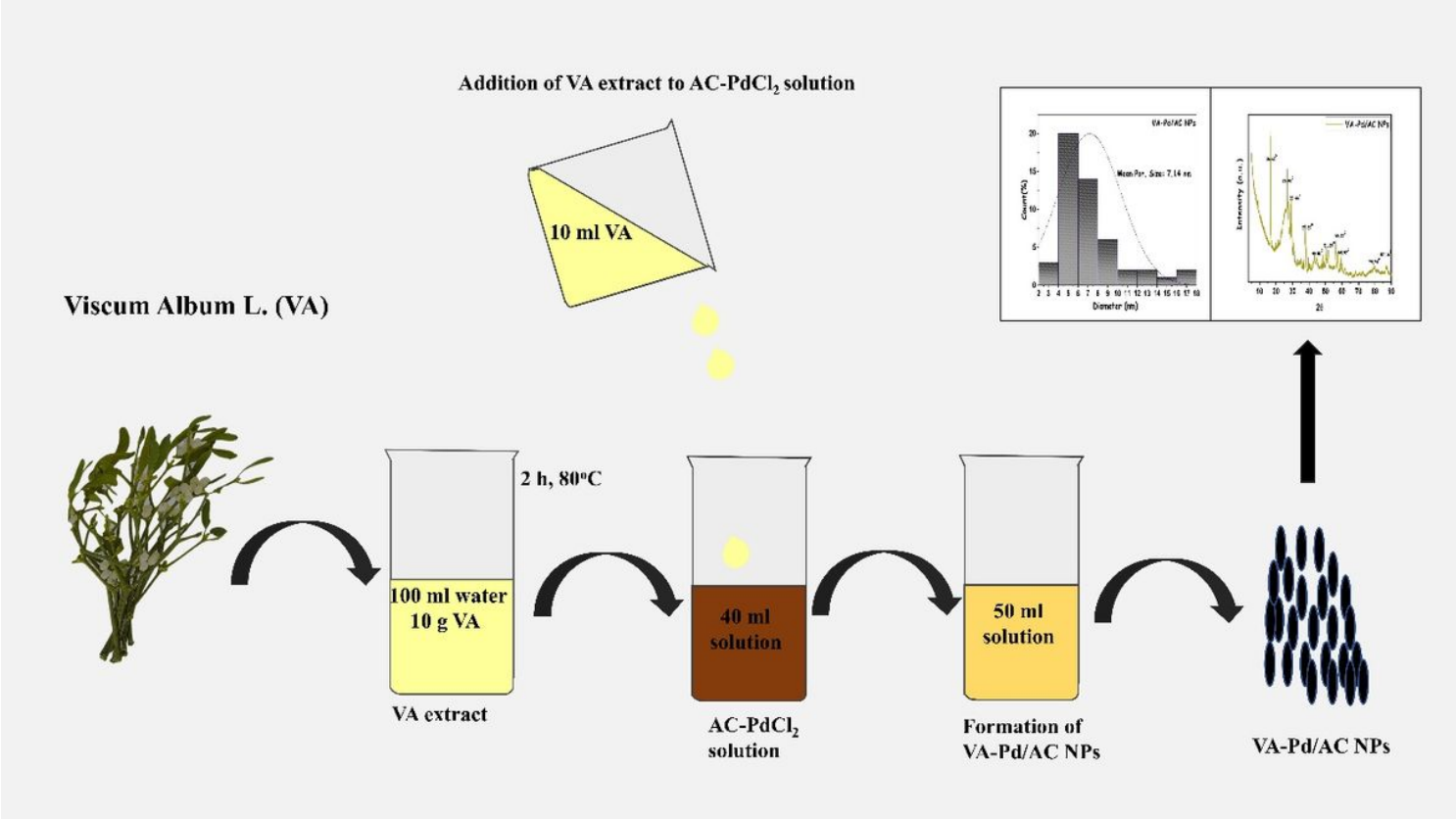


Figure 1

Green synthesis of palladium supported on activated carbon nanoparticles (VA-Pd/AC NPs) using the floral extract of Viscum album leaf as reducing and stabilizing agent

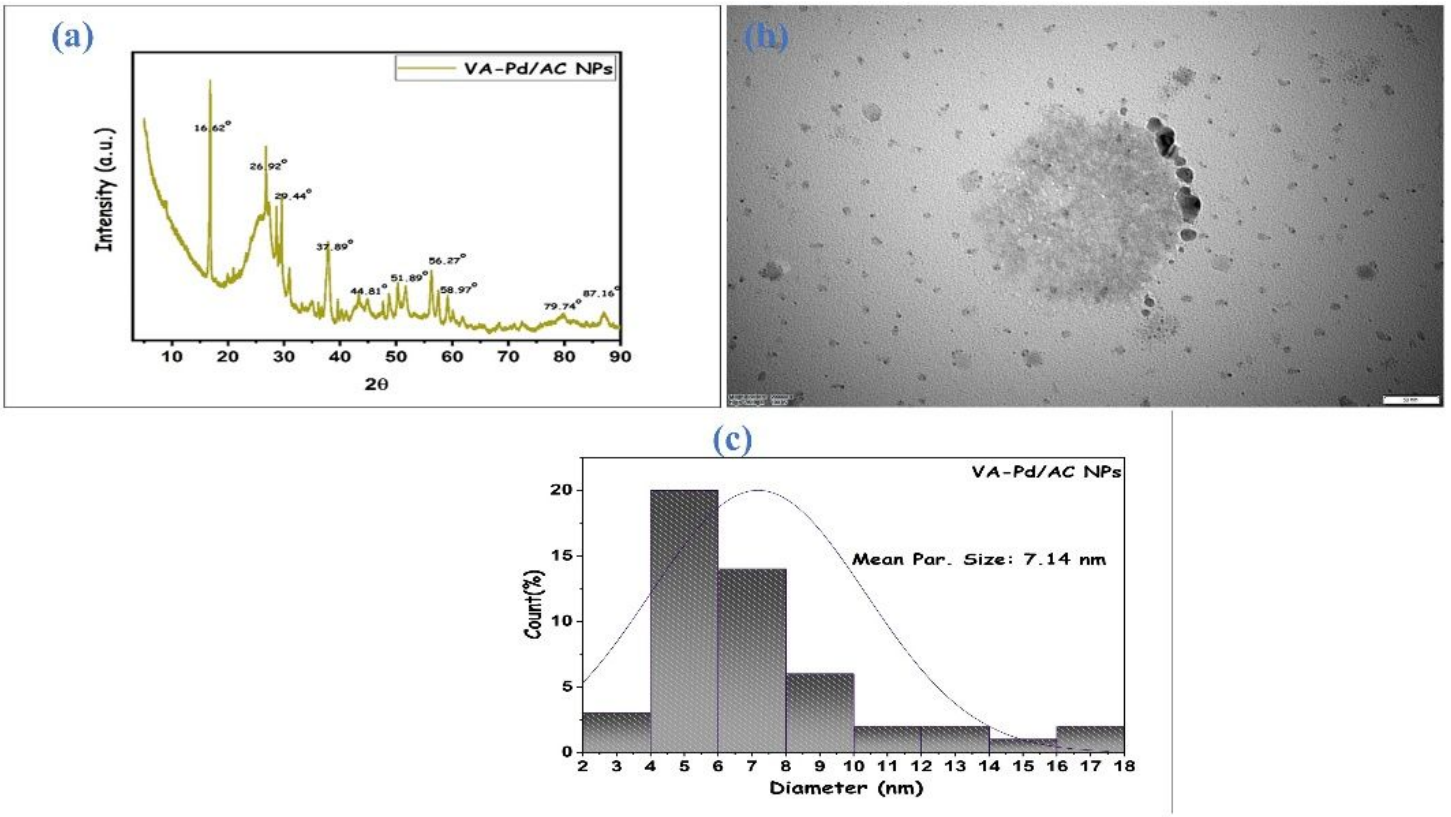


Figure 2

XRD analysis (a), TEM analyses (b) for metal particle distributions and particle histogram (c) obtained for VA-Pd/AC NPs

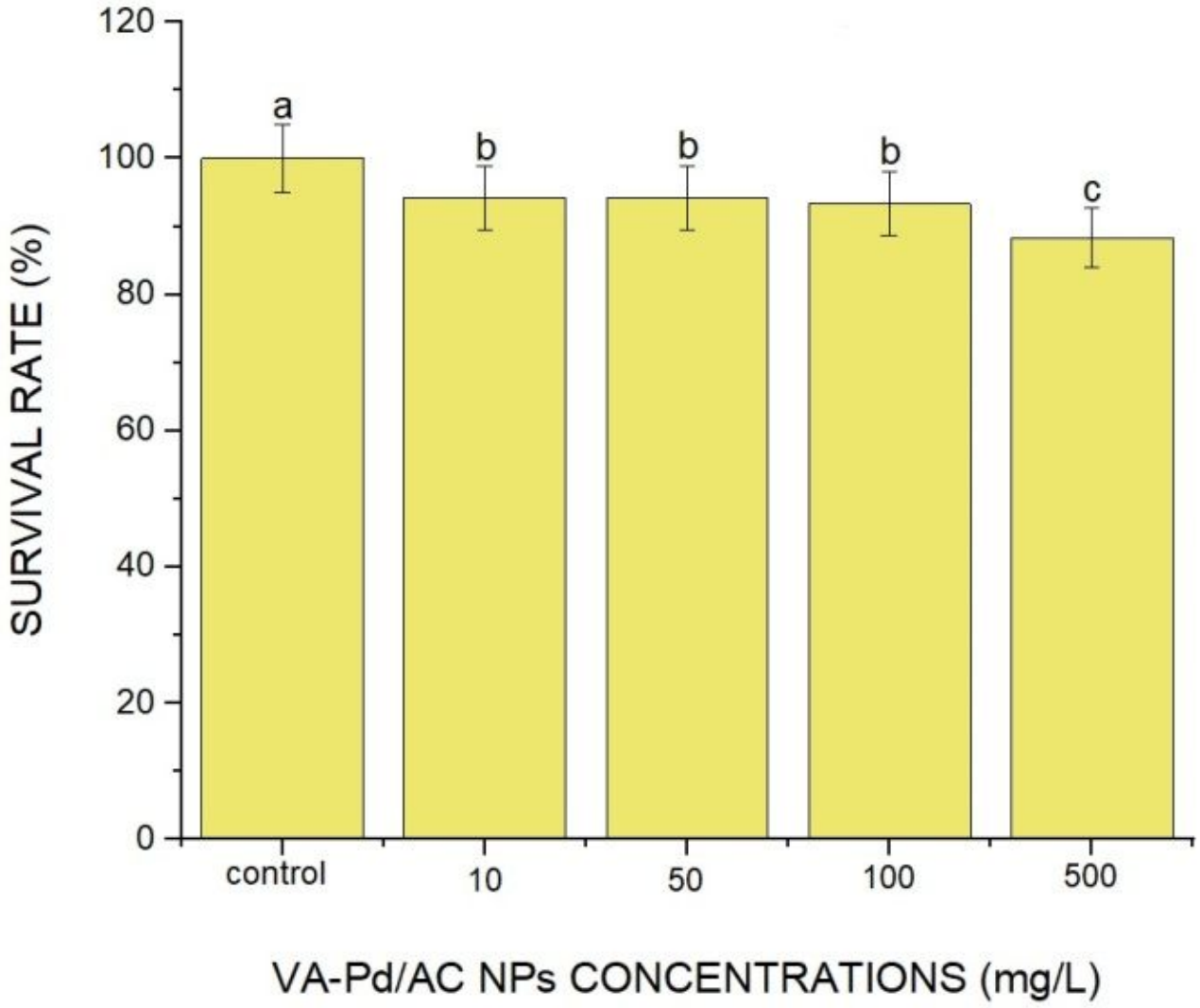


Figure 3

The dose-dependent survival effects of VA-Pd/AC NPs on zebrafish embryo/larvae at 96 hpf. Different letters indicate significant differences between the groups ($p < 0.05$) and each value is the average ± SEM.

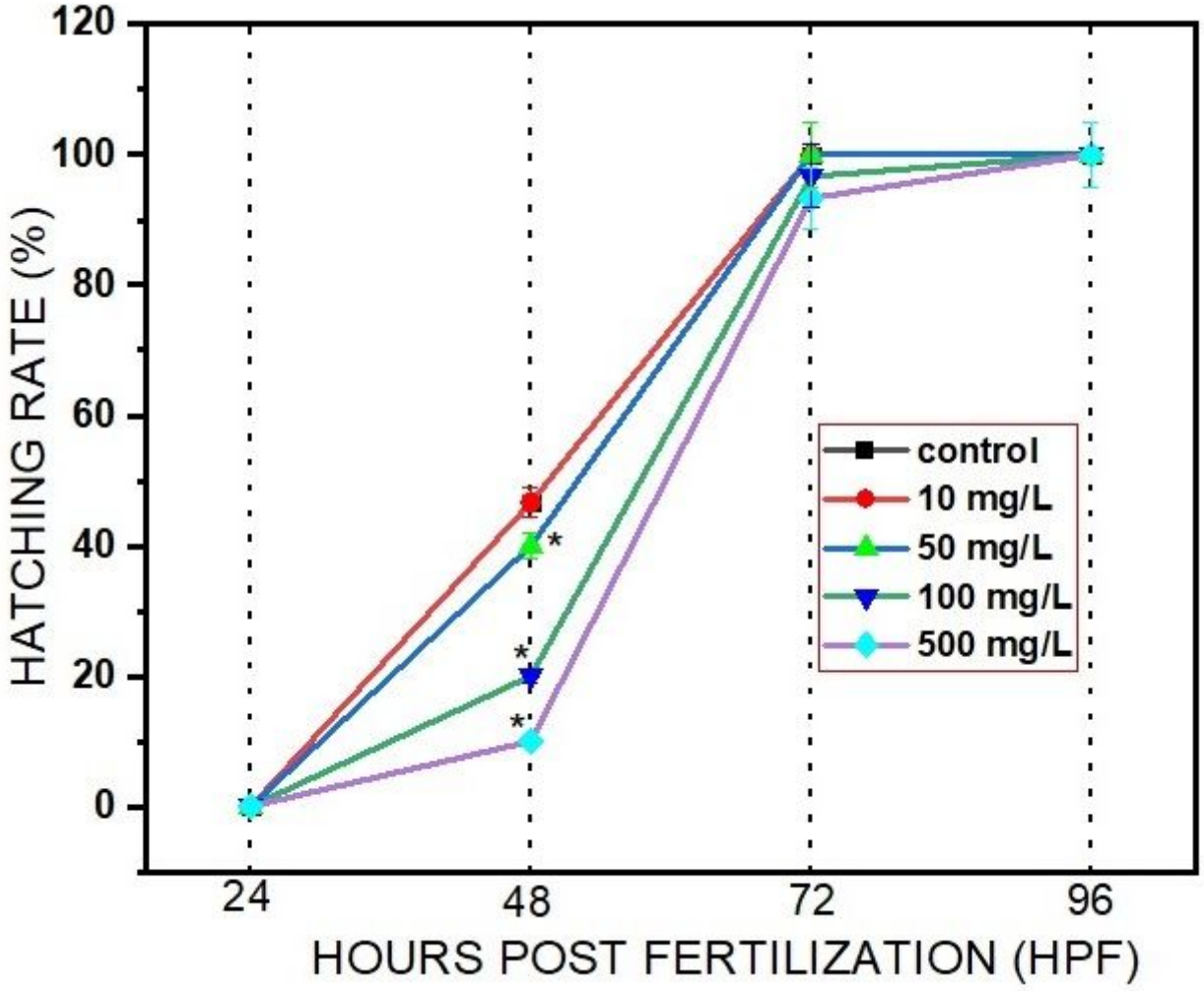
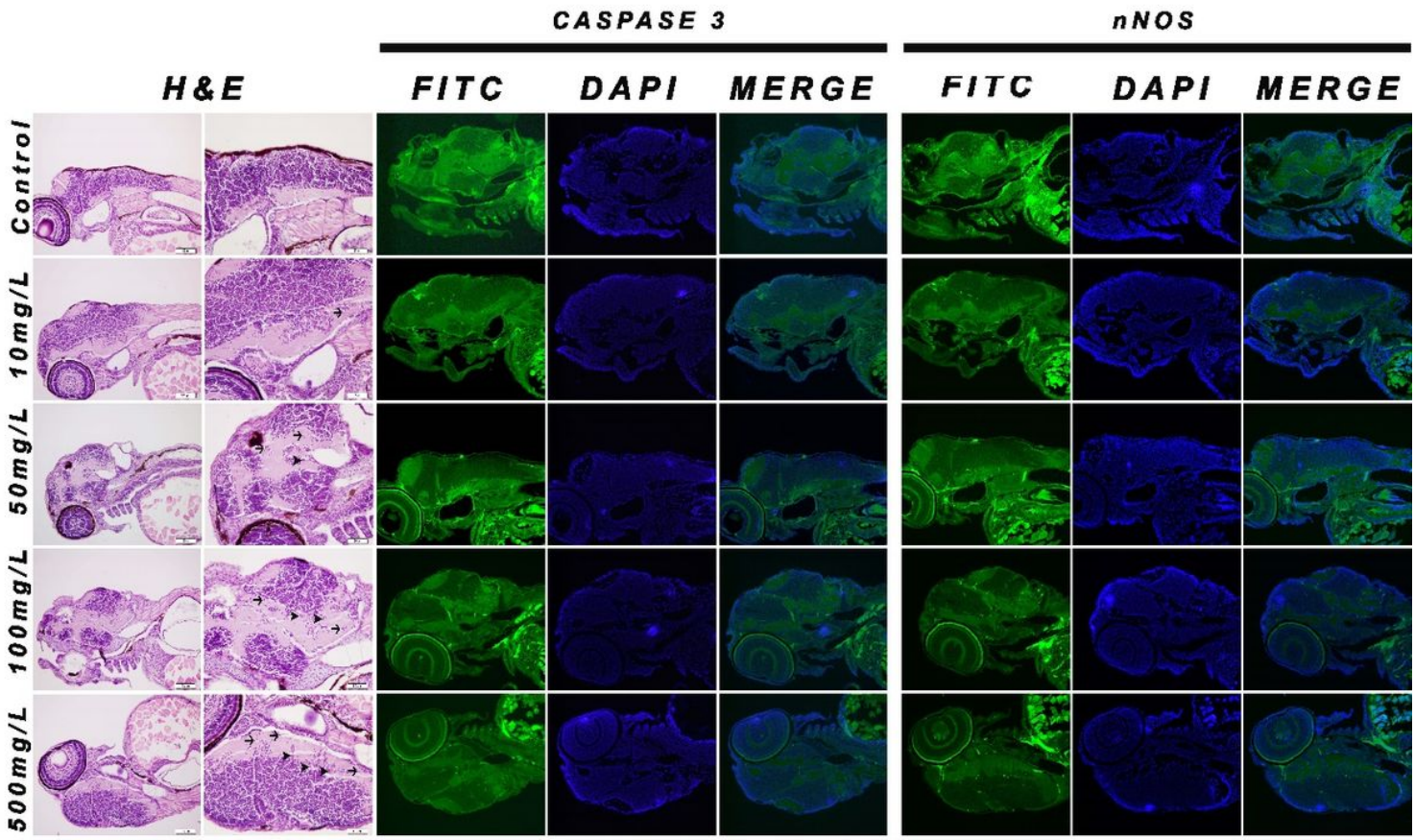


Figure 4

The hatching rate of zebrafish embryos exposed green syntheses VA-Pd/AC NPs from 24 to 96 hpf. *p < 0.05

Figure 5

Microscopic images of embryos malformations after VA-Pd/AC NPs exposure during 96 h. e) yolk sac edema and curved body axis.



	Control	10 mg/L	50 mg/L	100 mg/L	500 mg/L
Degeneration in neurons	-	-	+	++	+++
Necrosis in neurons	-	-	+	+	++
Caspase 3	-	-	+	++	+++
nNOS	-	-	++	++	+++

Figure 6

Representative immunhistochemical and immunofluorescence staining of caspase-3 and nNOS in control, 10 mg/L, 50 mg/L, 100 mg/L, and 500 mg/L of VA-Pd/AC NPs. Bar:100-50μm.