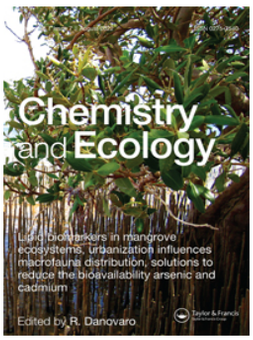

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Assessment of oxidative DNA damage, apoptosis and histopathological alterations on zebrafish exposed with green silver nanoparticle

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ABSTRACT

The effect of green synthesis approaches in NP synthesis and their possible toxicity in aquatic environments has been very limitedly studied. In this study, VA-Ag / AC NPs were synthesised using *Viscum album* plant as a reducing agent and *Chenopodium album* (CA) plant as an active carbon source. TEM (Transmission Electron Microscope) and XRD (X-ray crystallography) analysis of the synthesised VA-Ag / AC NPs were performed to detect their morphological and chemical properties. The inducing of oxidative stress and the effectiveness of VA-Ag/AC NPs in neurotoxic pathways and teratogenic changes in aquatic organisms have been investigated. In addition, a modelling was created to elucidate the toxicity mechanism. The results revealed that green synthesised VA-Ag/AC NPs nanoparticles are approximately 1600 times less toxic than nanoparticles synthesised by different methods. It had been determined that only high doses of VA-Ag/AC NPs nanoparticles cause neurological, histopathological and morphological changes. The findings in this study for VA-Ag/AC NPs, which are the evidence of the complexity of their mode of action at the cellular level, are a first in the aquatic ecosystem and require different findings regarding the stability and safety of such synthesis products.

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Zebra fish; bax; degeneration; necrosis; 8-OHdG; nanoparticle

1. Introduction

Recently, various plant-based approaches as a new alternative have been studied to avoid the limitations associated with the use of bacteria in the synthesis of nanoparticles [1]. In

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these approaches, plants are recognised as one of the richest sources for the green synthesis of nanomaterials [2]. The use of plant materials (such as leaves, stems, roots, fruits and flowers) in green synthesis makes a significant contribution to both non-toxic environmental friendly and covering or stabilising agents [3,4]. In the green synthesis of different sized and shaped Ag NPs various herbs are used, such as *Laminaria japonica, Azadirachta indica, Emblica officinalis, Tribulus terrestris, Pimpinella anisum*, *Chenopodium album*, *Taxus baccata, Trifolium resupinatum, Solanum melongena*, *Cardiospermum halicacabum, Olea europaea, Ziziphora tenuior, Justicia adhatoda, Nasturtium officinale, Syzygium cumini*, lemon, garlic, eucalyptus and aloe Vera [1,5,6].

The synthesis of biogenically Ag NPs using plant extract has a variety of important environmentally harmless and biomedical applications. For this reason, the most widely adopted method (among the biological methods) is phytogenic synthesis Ag NPs due to their special advantages such as wide distribution, easy availability and safety. There is a growing demand for green synthesis through green nanotechnology to avoid adverse effects in medical practices [4]. Therefore, scientists use plants as an alternative green synthesis approach due to their biological structure [6]. In nanoparticles synthesised by the integration of plant extracts, the essences play a role in different functions (reducing and/or stabilising). In this case, natural products such as flavonoids, terpenoids, carboxylic acid quinones, aldehydes, ketones and amides are effective in chelating metal products [5].

Chenopodium album is a leafy vegetable with a low shelf life and is used as a liquid extraction, reducing and stabilising agent. It is an excellent source of high-quality vitamins, proteins, nutrients and antioxidants, mainly retinol and ascorbic acid. Rich in fibre, *C. album* leaves are used in the treatment of different diseases [7]. One of the medicinal plants, the European mistletoe (*Viscum album* L.) is a parasitic, evergreen, perennial and epiphytic shrub that lives by absorbing the nutrients of different tree and shrub species. This plant, known in North America and Canada since the beginning of the twentieth century, has a wide spread in Europe and Turkey [5,8].

Album has been used in the biosynthesis of Ag NPs up to date [9–11]; however, no study has been found on its use in activated carbon production. However, there are very limited studies on the use of *V. album* on Ag NPs.

The zebrafish (*Danio rerio*) has become a famous vertebrate model in various biological disciplines. As a model organism with high genetic and physiological homology to humans, zebrafish is widely used in toxicological research and provides a solid basis for risk assessment of toxic substances. Thus, in many ways, the application of this model in toxicological studies will allow researchers to deal with the many challenges presented using other animal models, including sample size, inappropriate use, and higher monetary and time costs [12]. Zebrafish eggs are externally developed, it makes them easy to manipulate and suitable for high-yield applications. This is further augmented by the emerging zebrafish optical transparency, which allows for elegant visual analysis, including fluorescence and other markers. The growing of zebrafish is also incredibly fast, the basic zebrafish plan is well established by 24 h post fertilisation (hpf), embryogenesis is complete by 72 hpf, and most organs are fully developed by 96 hpf and reach adulthood in about 3 months. This makes them suitable for a wide variety of toxicological applications throughout their lifetime [13]. In recent years, Zebrafish (*Danio rerio*) as an in vivo model organism has attracted much attention due to its unique properties, including high fertility, embryo transparency, rapid and well-characterised development, low cost,

gene manipulation accessibility, short production time. Zebrafish embryo toxicity testing has become an important method for ecotoxicology research [14].

The effect of the extracts used in the synthesis of these products on the product mechanism (reducing and coating materials) had not been fully understood, although there have been studies on nanoparticles for a long time. However, how these nanoparticles affect the physiology and biology of aquatic organisms is another neglected area of research. This research has been performed to detect the importance of green nanoparticles and their widespread use. The teratogenic and neurotoxic damage potential of green particles (VA-Ag/AC NPs) formed by a different mechanism in zebrafish was investigated by considering the apoptosis pathway.

2. Material and method

2.1. Plants sample

Viscum album leaf used as a reducing agent was obtained at the border of the Erzurum region, Turkey. *Chenopodium album* (CA) used in the study was collected between the campus district boundaries of Iğdır University, Turkey. $AgNO_3$ was supplied by Sigma Aldrich company. The chemicals used in the study were of analytical grade and were consumed without any purification. All glass apparatus used were cleaned with distilled water and ethanol solvents.

2.2. Preparation of activated carbon

Chenopodium album (CA) is a plant species that widely known in the world is abundantly found in and around Iğdır University, in Turkey. It was used as a source of activated carbon. The harvested CA plant was left to be preserved in the oven at 80°C for 24 h after a series of washing operations. Thereafter, it was milled in size ranges of 150–250 µm. Carbonisation process of the *C. album* (CA) samples was carried out at a heating rate of 5°C min^{-1} for 120 min at 600°C in a sample chamber under nitrogen flow atmosphere. Thereafter, the sample was allowed to cool to room temperature (RT), the final storage procedure was applied according to Kokturk et al. [15].

2.3. Viscum album extracts preparation

In the study, *Viscum album* (VA) extract was used as a reducing agent. Before the experiments, *V. album* plants were cleaned using abundant distilled water to remove impurities. Then, kept in an oven to dry at 105°C for 24 h. The dried VA was ground into very small pieces. 100 mL of deionised water was added to 10 g of VA. The suspension mixture was boiled at 80°C for 2 h. The temperature of the VA extract was allowed to cool to room temperature, was filtered under vacuum. In this study, the dose determination, especially for *Viscum album* [16], the toxicity concentrations of *V. album* aqueous extracts in cell cultures (100 mg/L were found to have no toxic effect) and the LC_{50} were carried out according to our previous studies. Taking into account the VA-Ag/AC NPs NPs sublethal doses of 100 mg/L and lower values were selected. In addition, while the concentration in which the aqueous extract of the *V. album* plant was used in the synthesis of nanoparticles was prepared, and 100 mL/10 gr doses were used.

2.4. Synthesis of VA-Ag/AC NPs

The synthesised products' instrumental analyzes were performed according to Calımlı [17] and Kokturk et al. [15] (Figure 1(I)). For the green synthesis process of VA-Ag/AC NPs, 0.02 g $AgNO_3$, and 0.02 g AC particles were dissolved in 40 mL of distilled water, and vigorously stirred at room conditions. Thereafter, 10 mL of VA extract was added to this mixture and placed in a 250 mL glass vial and subjected to vigorous stirring at the same condition. Prepared VA-Ag/AC NPs were separated by centrifugation at 12,000 rpm for 15 min. The separated VA-Ag/AC NPs were washed with distilled water

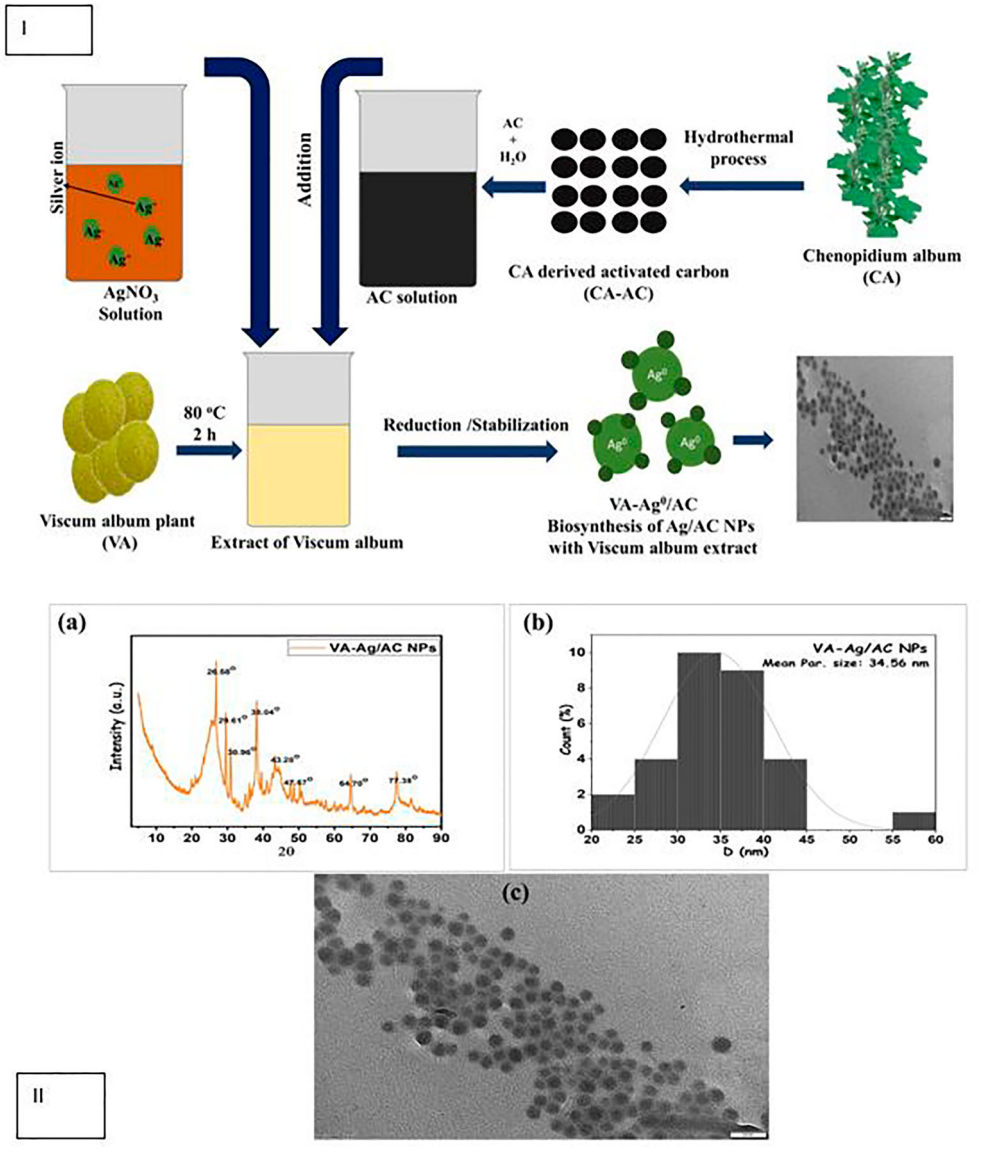

Figure 1. (I) Green synthesis mechanism process for producing VA-Ag/AC NPs, (II) XRD analisis (a), mean particle size (b) and TEM image (c) of VA-Ag/AC NPs.

and ethanol and dried in a vacuum oven at 60°C for 2 h and stored for the characterisation and experimental application studies.

2.5. Determination of pHzpc values of AC and VA-Ag/AC

The pH values of zero point of charge (pHzpc) of AC and VA-Ag/AC were determined using a method as described previously [18]. Briefly, a 20 mL 0.01M NaCl solution was prepared. The pH values of these solutions were adjusted from 2 to 12 using NaOH and HCl. 20 mg of AC was added to 20 mL of 0.01 M NaCl solution. Then, different pH (2–11) values of the obtained solution were adjusted using 0.1 M NaOH and HCl chemicals. The final pH values of each suspension were recorded after 6 h. Similarly, the pH value of the zero point charge (pHzpc) of VA-Ag/AC Ns was prepared following the steps of the above-mentioned AC Ns zero point charge (pHzpc) preparation procedure.

2.6. In vivo study

2.6.1. Zebrafish

Wild-type AB adult zebrafish (*Danio rerio*) for the trial, were obtained from Izmir Biomedicine and Genome Center Zebrafish Center. The research conditions, fish environments, and the experimental setup were designed according to as described elsewhere Kokturk et al. [15].

2.6.2. Determination of LC_{50} and survival rate, hatching rate, and embryo morphological changes

According to adult zebrafish, optimal water conditions were organised as: the water temperature 28°C, pH 7,2 with a 14-h light/10-h dark cycle. The study was planned according to a semi-static test using 4 repeats [19]. The experiment consisted of 5 groups with 30 embryos each: one control and four repeats of treatment. A stock solution (VA-Ag/AC NPs; 2000 mg/L) was prepared for the determination of LC_{50} values. The dilution was performed for the determined concentrations (10, 50, 100 and 500 mg/L) in E3 embryo medium (0.17 mM KCl, 0.33 mM $MgSO_4$, 5 mM NaCl, and 0.33 mM $CaCl_2$). Having no heart rate embryos up to 24 hpf and after were recorded as dead embryos. The count of dead embryos was counted at periodic intervals and immediately removed from the experimental setup. Mortality rate was determined after 24, 48, 72 and 96 h. Acute toxicity tests were conducted according to the standard methods [15]. The embryos of the treatment groups, which were designed with reference to the determined LC 50 value, were screened for 24–96 h (SZX16 Olympus stereomicroscope with SC50 Olympus camera), and survival rate, hatching rate, and embryo morphological changes were observed.

2.7. Histopathological and immunofluorescence assays

Larvae samples were prepared considering the routine tissue collection and experiment procedure diagram of Kokturk et al. [15]. The obtained histopathological findings were interpreted using a simple scoring technique [none (–), mild (+), moderate (++) and severe (+++) according to immune positivity]. In the immunohistochemistry analyses performed in the same study, it was a routine procedure with minor modifications [Primary

antibodies (8-OHdG, Cat No: sc-66036, Diluent Ratio: 1/100, Bax Cat No: sc-7480 Diluent Ratio: 1/100, Santa Cruz)] followed by fluorescence microscope imaging. The images were interpreted with the scoring used in histopathology.

2.8. Statistical analyses

Parametric assumptions for comparisons were studied using SPSS for Windows 20.0 and with one-way analysis of variance (ANOVA). To test whether there was a statistically significant difference between the means of independent groups, One-way ANOVA test was used. Conditions such as obtained data's conformity to normal distribution and equality of group variances were effective in the selection of this test. The originates of difference were determined with Tukey and Duncan tests. ($p < 0.05$). Kruskal–Wallis test was used for semi-quantitative non-parametric findings and Mann–Whitney U test was used for comparison of paired groups.

3. Results and discussion

3.1. Characterisation of VA-Ag/AC NPs

XRD analysis for VA-Ag/AC NPs is given in Figure 1(II)(a). Significant peaks at 26.58°, 43.28° and 47.67° values in XRD analysis correspond to carbon atoms as stated in the literature [20]. The peaks of 37.08°, 43.28°, 64.70° and 77.38° for Ag atoms are in line with the literature [21] with few deviations. This shows the presence of silver atoms in the particle crystal structure obtained. These peaks correspond to the crystal fringes 111, 200, 220 and 311. TEM analysis and calculated particle histogram are given in Figure 1(II)(b,c) for VA-Ag/AC NPs, respectively. The average particle size of VA-Ag/AC NPs was calculated as 34.56 nm by counting approximately 50 spherical particles and using Imagej program.

The pHpzc values for activated carbon and VA-Ag/AC NPs were determined as 6.96. and 7.62, respectively . The obtained pHpzc values are shown in Figure 2 by plotting pH versus ΔpH.

VA-Ag/C NPs nanoparticle shapes, as seen in TEM analyzes, were spherical in density. Similarly, mixed-phase (hexagonal and cubic) syntheses had been reported in different Ag nanoparticle studies [22]. However, the shift of the X-ray diffractogram deviations in green synthesis particles (VA-Ag/AC NPs) to higher values compared to other studies can be explained by the interaction effect between herbal extracts and Ag-NPs [23]. In addition, considering the studies of Kaabipour and Hemmati [24], basic reaction parameters such as temperature, pH, precursor and reagent are thought to be effective in the difference in VA-Ag/C NPs particle size (34.56 nm) compared to other studies.

3.2. Determination of LC_{50} value at 96 hpf

VA-Ag/AC NPs LC_{50} 96 value was determined to be 127.79 mg/L in the present research. 48-hour LC_{50} value of Ag NPs (synthesised at 81 nm size) was recorded as 84 µg L^{-1} (0.084 mg / L) for zebrafish, in a previous study [25]. It is thought that the less toxicity of synthesised VA-Ag/AC nanoparticles compared to Ag-NPs is associated with a lower dissolution rate coefficient [26]. However, in the toxicity mechanism, the effect of used

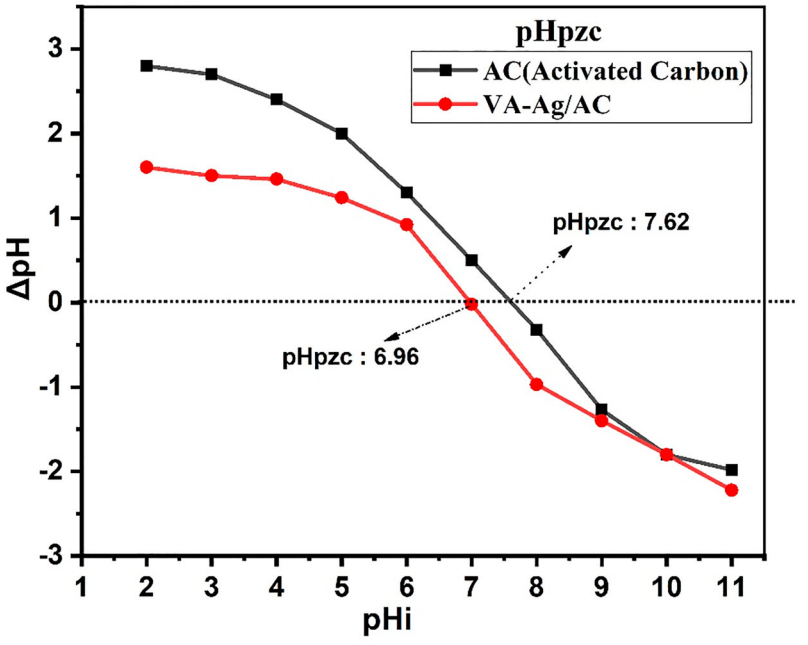

Figure 2. Point zero charge of (pHpzc) of AC and VA-Ag/AC nanoparticle (0.01 M NaCl, 25°C, 600 rpm, 6 h).

plant extracts may have played a role. If we compare with Bilberg et al. [25], the LC50 value of VA-Ag/AC NPs, which we synthesised in almost half the size, is both emerging in 96 h and being 1600 times less toxic makes green synthesis privileges VA-Ag/AC NPs among Ag NPs. AgNPs can also form intermolecular coupling with the Ag^+ ion, resulting in a large negative charge and reduced toxic effects [27].

3.3. Assay of effect of VA-Ag/AC NPs on survival rate, hatching, and malformation

Survival was found to be significantly reduced in embryos/larvae of treatment groups exposed to different concentrations (10, 50 and 100 mg/L) of VA-Ag/AC NPs. Although this decrease followed a concentration-related course (Figure 4, $p < 0.05$), the difference between the groups was insignificant when compared with the control group (Figure 3 (A), $p > 0.05$).

The presence of Ag^+ in solution media has an impact on the hatching or survival of zebrafish embryos [28]. In another study, it was reported that the oxidative environment around nanoparticles may cause Ag^+ release and also adverse effects in aquatic organisms [26]. For freshwater fish, ion balance disturbances caused by silver ions on Na–Cl ions can have fatal consequences for fish [25].

When the effects of VA-Ag/AC nanoparticles on the zebrafish larval hatching rate were examined, it was observed that there was a significant difference between all treatment groups (10, 50 and 100 mg/L) and the control group at 48th and 72nd hours (Figure 3(B), $p < 0.05$). The lowest larvae hatching rate was found to be 11% and 8% in the 50 and

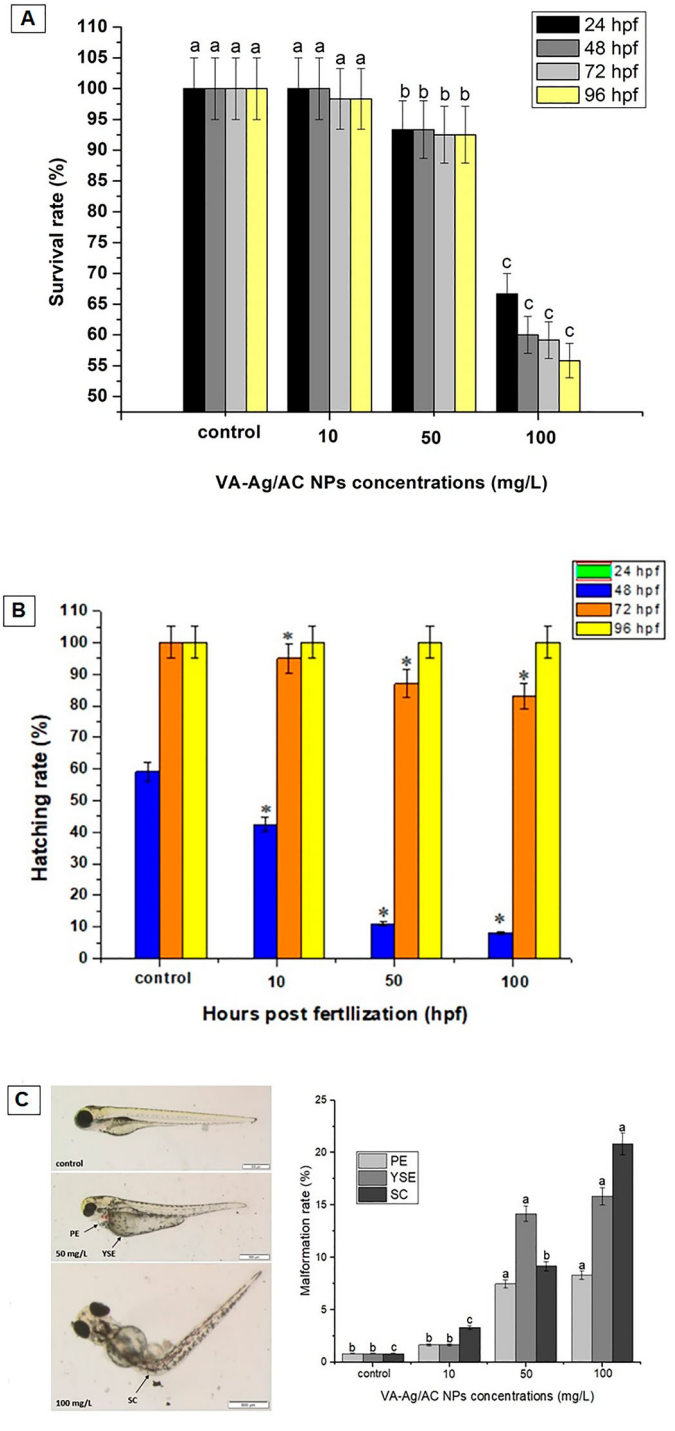

Figure 3. (A): Effects of VA-Ag/AC NPs on zebrafish embryo/larvae survival at 24, 48, 72, and 96 hpf. (B): Effects of VA-Ag/AC NPs on hatching rate of zebrafish embryos (C): Effects of VA-Ag/AC NPs on Microscopic images of embryos and percentage of observed malformations (Scale bar: 500 µm) (PE: pericardial edema; YSE: yolk sac edema; SC: spinal curvature). Different letters indicate significant differences between the groups ($p < 0.05$) and each value is the average ± SD. Scale bar: 500 µm.

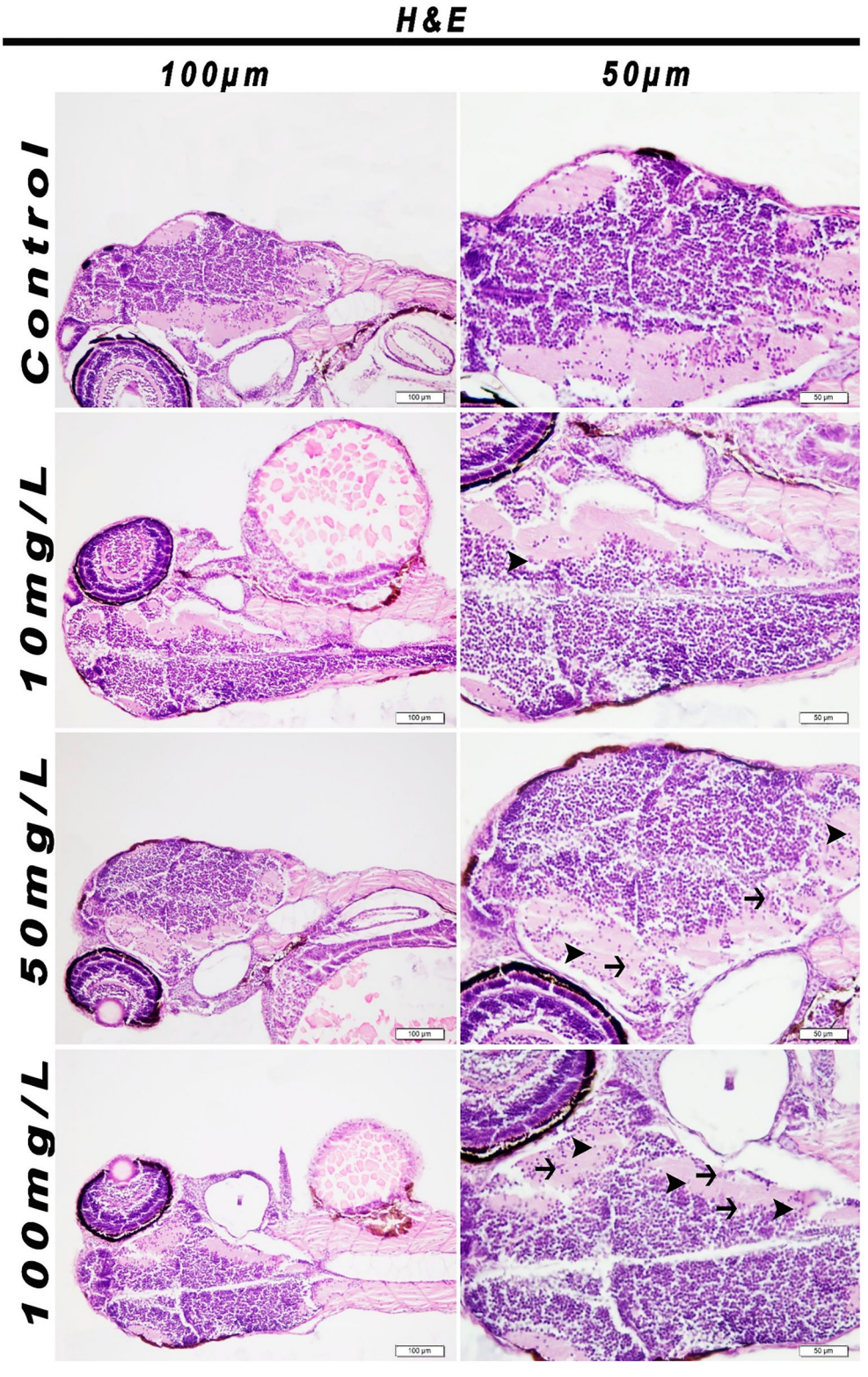

Figure 4. Control group, normal histological appearance, 10 mg/L group, mild degeneration in neurons (arrow head), 50 mg/L group, moderate degeneration in neurons (arrow heads) and moderate necrosis (arrows), 100 mg/L group, severe degeneration in neurons (arrow heads) and severe necrosis (arrows), H&E, Bar: 100–50 µm.

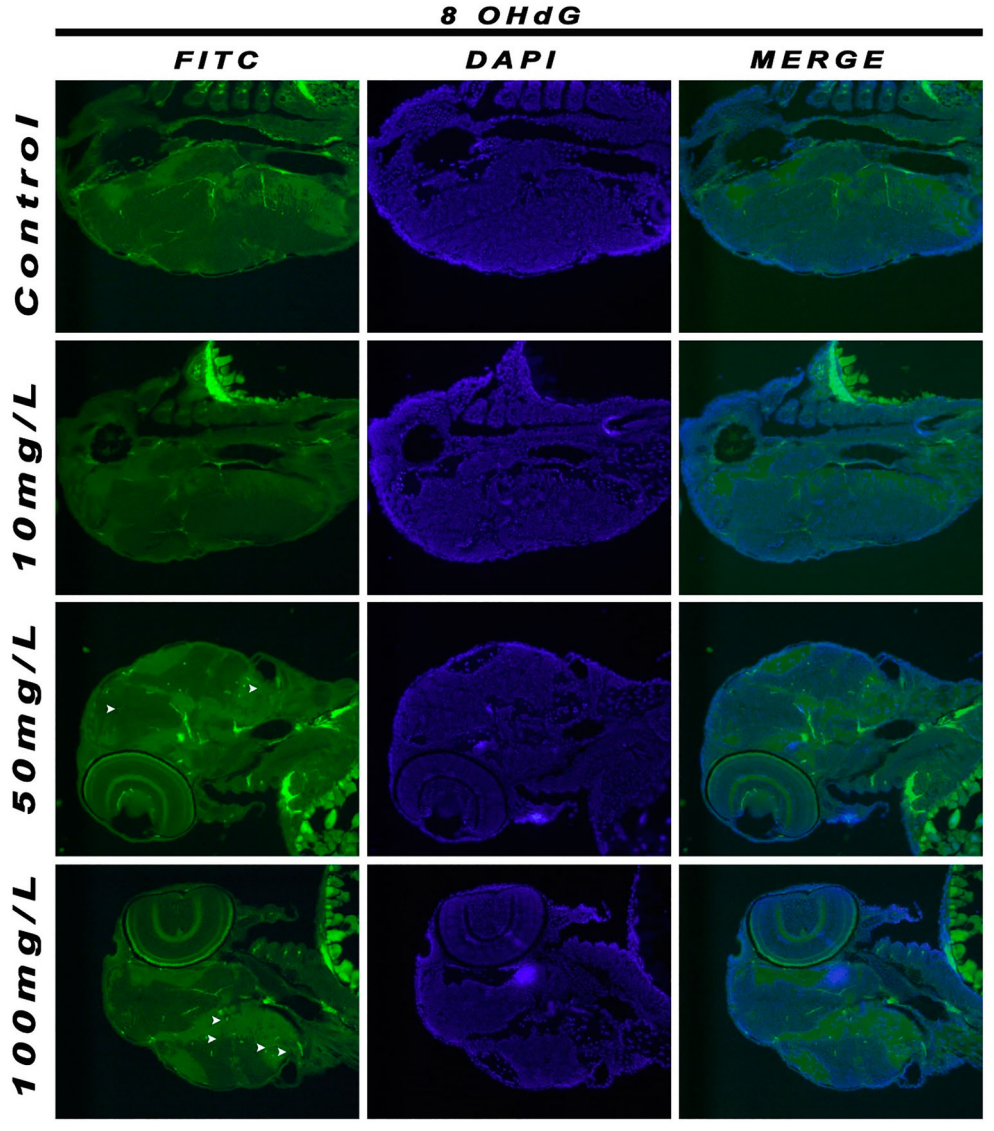

Figure 5. Control and 10 mg/L groups, negative 8-OHdG expression, 50 mg/L group, moderate expression of 8-OHdG in neurons (arrow heads), 100 mg/L group, severe expression of 8-OHdG in neurons (arrow heads), IF, Bar: 50 µm.

100 mg/L groups at 48th hour, respectively. The differences in toxicity between groups may have been caused by Ag^{+} release rates and/or plant concentration levels. Accordingly, the size of Ag NPs and the difference of stabilising agents, different exposure times and organisms affect their toxicity degree [25].

Zebrafish embryos have a chorion that acts as a protective barrier against toxic substances. This structure, which has pores of 0.5–0.7 µm in diameter, is responsible for transporting oxygen and nutrients for embryos [29,30]. This is an opportunity for uptake of AgNPs. The AgNPs entering into chorion is effective in embryo tissues and/or later

developmental abnormalities [30]. Qiang et al. [30], had examined the effect of different particle sizes (from 3.7 to 5.7 nm and from 7.9 to 14.5 nm) on toxicity. In this study, we reported that a smaller sized NPs. The smaller sized Ag NPs (which obtained by us) were more effective in adhesion to the embryo chorion. This caused developmental toxicity by blocking the chorion pores and inhibiting oxygen exchange. In addition, morphological defects (such as yolk sac edema, pericardial edema, and spinal curvature) were observed in all groups exposed to VA-Ag/AC NPs (Figure 3(C)). The highest morphological abnormality as spinal curvature (21%) was determined in the 100 mg/L group (Figure 6).

All of these malformations were observed at significant rates between control group and 50 and 100 mg / L groups (Figure 6, $p < 0.05$). It is thought that the particle size

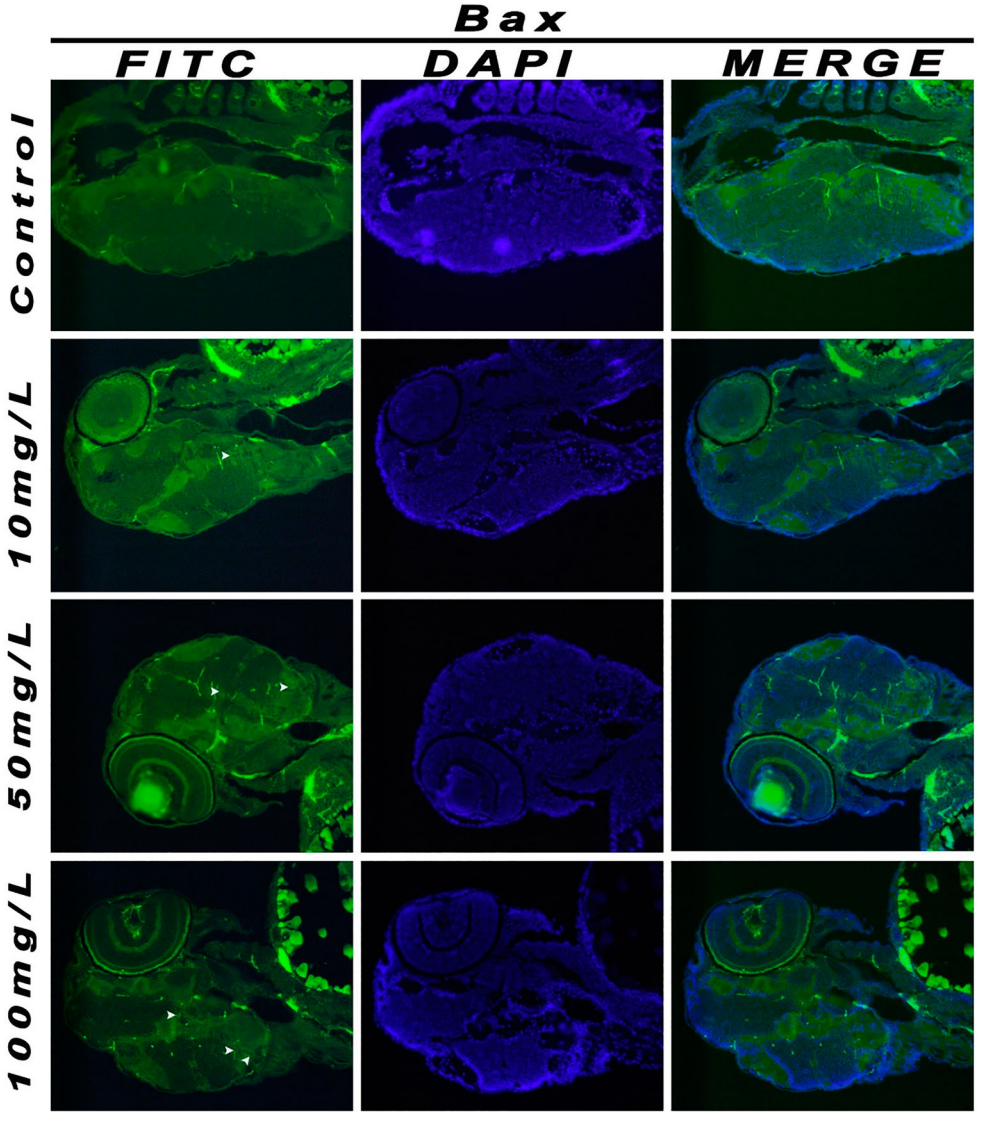

Figure 6. Bax expression in neurons of zebrafish (arrow heads), Bar: 50 µm.

(34.56 nm) is as effective as the herbal extract used in the synthesised VA-Ag/AC NPs in these toxicity differences (having a milder course of toxicity compared to similar studies). Once Ag NPs are released into the natural aquatic environment, they suffer from physicochemical transformations in terms of aggregation and chemical speciation that have major effects on their transport, fate and toxicity. This transformation depends on the environment in which the NPs are released, as well as on their intrinsic properties such as surface area, shape, capping composition, charge, among other properties [27]. It has been reported that small particles have higher dissolution rates than large particles (due to increased surface area) and are more efficient carriers for local release and accumulation of Ag ions in the fish body [30].

By comparative analysis, there are fourteen impaired metabolic pathways, related to phosphorus assimilation under Ag NP exposures. Among them, five metabolic pathways were significantly affected, including inositol phosphate metabolism, phosphatidylinositol signalling system, and glutathione metabolism in algal cells. Moreover, enrichment analysis showed that AgNPs significantly affected metabolic pathways related to carbohydrate metabolism, amino acid metabolism and fatty acid metabolism [31]. These pathways are closely related to cellular functions such as cell growth, migration and differentiation, and disruptions in these mechanisms lead to strong adverse effects [26]. In our study findings, it is thought that the determined for VA-Ag/AC NPs toxicity is mainly caused by dissolved Ag +.

Any silver bioaccumulation observed in embryos could be due to both Ag NPs and free silver ions. Qiang et al., [30] reported that exposure to Ag NPs and/or silver ions alone can lead to similar phenotypic abnormalities such as edema formation. This phenomenon supports our current research finding.

3.4. Assay of effect of VA-Ag/AC NPs on toxicity pathway (histopathological and immunofluorescence findings)

In the histopathological and immunofluorescent analyzes, normal histological appearance was observed in the larvae of the control group. A mild degeneration of neurophils was observed in 10 mg/L application group; moderate degeneration and necrosis were detected in the neurinophiles at 50 mg/L group; and severe degeneration and necrosis in the 100 mg/L group (Figure 4, Table 1). These differences were statistically significant compared to the control group ($p < 0.05$).

Molecules such as calcium, ceramide and Bcl-2 family, organelles such as p53, caspases and mitochondria are involved in the regulation of apoptosis. The Bcl-2 gene family has the most important role in regulating apoptosis.

The Bcl-2 family includes proapoptotic proteins Bid, BclXs, Noxa, Bad, Bax, Bim, Puma, and Bak. Among these proteins, Bax is the cofactor of the repressor protein of p53. Bax is

Table 1. Scoring of histopathological and immunofluorescence findings of zebrafish larvae samples.

	Control	10 mg/L	50 mg/L	100 mg/L
Degeneration in neurophils	–	+	++	+++
Necrosis in neurophils	–	–	++	+++
8-OHdG	–	–	++	+++
Bax	–	+	++	+++

induced by p53, stimulates apoptosis via the intrinsic pathway and accelerates the cell's progression to apoptosis [32,33].

At high AgNP concentrations, cells undergo necrosis as they are unable to overcome irreversible damage [34]. This supports our research findings for VA-Ag/AC NPs administered at high doses. At low concentrations, we can say that it tolerates NP toxicity through the activation of cell death modalities such as apoptosis as an attempt to overcome it.

Zebrafish larvae samples were examined as immunofluorescence method. **Control group** the expression of 8-OHdG and Bax was evaluated as negative. **10 mg/L group** 8-OHdG expression was detected as negative, and mild Bax expression was detected in neurophils. **50 mg/L group** moderate level of 8-OHdG and Bax expression was detected in neurophils, **100 mg/L group** severe 8-OHdG and Bax expression was detected in neurophils (Figures 5 and 6). A statistically significant difference ($p < 0.05$) was found when compared with the control group. Based on our research findings, we can say that DNA damage triggers apoptosis. However, Saud Alarif et al. [35] reported that induction of apoptosis or necrosis can occur indirectly through interaction with oxygen radicals or other reactive intermediates or as a result of excision repair enzymes.

In a healthy organism, reactive oxygen species (ROS) can also be formed during normal metabolism. Factors such as inflammation, drugs, exogenous sources and exposure to radiation encourage and increase this formation. Oxidative damage causes nitration or deamination of DNA bases. Cell death (apoptosis) occurs when oxidative damage in DNA reaches high [36].

In the current study, it is thought that this process may have developed under the influence of silver, and that NP may spontaneously cause the formation of free radicals and cause reactive formation due to surface properties and/or surface oxygen species [35].

Our research findings are consistent with previous toxicity studies that reported an increase in Bax protein expression depending on the level of damage in brain tissue [37–39]. Based on the present results and other reports, we can say that such bioindicators have the potential to serve as markers of NPs-induced apoptosis [40]. Finally, low doses of VA and CA extracts used in green synthesis exert neuroprotection and are effective in interpreting the apoptosis process, which is inhibited by decreased Bax levels. Because Bax increases the permeability of mitochondrial membranes, causes the release of cytochrome C and plays an important role in inducing apoptosis with the activation of caspase-3 and 9 [41].

4. Conclusion

VA-Ag/AC NPs, a green synthesis product (of Ag NPs with a combination of *Viscum album* leaf extract and silver-activated carbon materials), had shown many advantages such as safe, non-toxic and environmental friendly synthesis. The present study's results show that the VA leaf aqueous extract can act as a reducing agent as well as a capping agent, which can be used for the synthesis of Ag NPs. Structural characterisation (by XRD and TEM analyses) of VA-Ag/AC NPs, mean particle size (34.56 nm) and shape (spherical) of Green synthesis VA-Ag/AC NPs were confirmed.

However, the examinations over of brain tissues by histopathological and immunofluorescence methods determined that degeneration, necrosis, 8-OHdG and Bax expressions in neurons increased depending on concentration.

The overall results prove that *V. album* leaf extract is an important alternative to the traditional chemical reduction method to avoid chemical use. From the teratogenic and neurotoxic results, it was concluded that different concentrations of VA-Ag/AC NPs may be the cause of the (but also different) morphological changes shaped by oxidative damage in zebrafish.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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