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Investigation of the Oxidative Stress Response of a Green Synthesis Nanoparticle (RP-Ag/ACNPs) in Zebrafish

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Abstract

Silver nanoparticles (AgNPs) are prominent nanomaterials that are efficiently used in different industries including medical products, water treatment, and cosmetics. However, AgNPs are known to cause adverse effects on the ecosystem and human health. In this study, aqueous extract of *Rumex patientia* (RP) was used as a reducing and stabilizing agent in AgNP biosynthesis. The obtained activated carbon (AC) from *Chenopodium album* (CA) plant was combined with RP-AgNPs to synthesize RP-Ag/AC NPs. Next, the effects of these green synthesis RP-Ag/AC NPs on zebrafish (*Danio rerio*) embryos and larvae were investigated. First, we characterized the RP-Ag/AC NPs by using X-ray diffraction (XRD) and transmission electron microscopy (TEM) and determined LC_{50} value as 217.23 mg/L at 96 h. Next, the alterations in survival rate, hatching rate, and morphology of the larvae at 96 h were monitored. The survival rates decreased in a dose-dependent manner. Morphological defects such as yolk sac edema, pericardial edema, spinal curvature, and tail malformation in the NP-treated larvae were observed. RP-Ag/AC NPs stimulated the production of neuronal NOS (nNOS) and 8-OHdG in zebrafish brain tissues in a dose-dependent manner and enhanced neutrophil degeneration and necrosis at concentrations of 50 and 100 mg/L. Thus, the obtained data suggest that the green synthesis process is not sufficient to reduce the effect of oxidative stress caused by AgNPs on oxidative signaling.

Keywords *Danio rerio* · Nanoparticle · nNOS · 8-OHdG · Morphological defects · Survival rates · Patience dock · Goosefoot

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Introduction

While nanotechnology-related research is rapidly growing, there are some concerns about the possible risks and effects of nanoparticles (NPs) on the environment and human health [1]. These concerns lead to the development of environment-friendly green synthesis methods that have many advantages such as efficient particle generation, high success, low cost, and ease of characterization [2]. Green synthesis process has been realized by reduction of the use or production of raw materials, products, by-products, solvents, and reagents that can significantly impair human health and the environment [3–5]. Green synthesis products are notable for their potential to reduce the toxicity of NPs. The usage of vitamins, amino acids, and plant extracts is becoming very popular today in this technology [6]. The plant-based NP synthesis procedure is easier. A metal salt is synthesized with the plant extract and the green NP synthesis is completed in a short time (from minutes to several hours) at room temperature

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[7]. Recently, this strategy has received more attention, especially for silver (Ag) and gold (Au) NPs, which are safer compared to other metallic NPs [8–11].

The distinctive plasmonic properties of nanomaterials enable them to have interactions with a wide spectrum of biomolecules [12]. Therefore, NPs can be used for diagnosis or treatment. Previous research has suggested oxidative or nitrosative stress associated with several NPs. AgNPs have also been shown to have antimicrobial properties [13]. Their combination with plant extracts can create AgNPs with enhanced bioactive properties. Therefore, plant-based AgNPs can exhibit higher biological activities than traditional products [12].

Chenopodium album (CA) is a widely distributed plant species that contains various bioactive constituents with potential therapeutic activities [14]. However, the effect of different extraction techniques for the CA on the secondary metabolites' concentration (flavonoids, tannins, saponins, and alkaloids) remains to be investigated [14]. *Rumex patientia* (RP) may have beneficial effects on metabolic diseases [15]. The aqueous extract of the RP roots exhibited high anti-inflammatory activity [16]. NPs can cause stress on organisms due to toxic metabolites [17, 18]. The toxic effects of any chemical on tissues and cells can be examined using various methods. For example, the response of organisms to chemicals can be evaluated dynamically on fish tissue and organ systems in vivo [19–24]. Zebrafish is a frequently used vertebrate model to monitor morphological changes, survival rate, oxidative stress response, immunological processes, DNA damage, and apoptosis caused by various chemicals [19–21, 25, 26]. In this study, we investigated the biocompatibility and toxicity of green synthesis RP-Ag/AC NPs obtained from RP aqueous extract in the zebrafish larvae. RP-Ag/AC NPs not only reduced the survival and hatching rate of the zebrafish larvae but also caused morphological defects and neurotoxicity as determined by histopathological and immunohistochemical analyses. Thus, it is essential to perform the risk assessment of health and environmental effects of the products obtained by the green synthesis process.

Materials and Methods

Plant Sample

Patience dock (*Rumex patientia* (RP)) was obtained at the border of the İzmir region, Turkey, and its leaf used as a reducing agent. Goosefoot (*Chenopodium album* (CA)) was collected on the slopes of Mount Ağrı, Turkey. $AgNO_3$ was supplied by Sigma-Aldrich Company. The used glass materials of analytical grade were consumed without any purification.

Preparation and Characterization of RP-Ag/AC NPs

Preparation of Activated Carbon

Chenopodium album (CA) is a widely known and abundant plant species around the world. CA was used as the source of activated carbon (AC). Preparation of AC was carried out as described elsewhere [27]. The harvested CA plant was incubated in an oven at 75 °C for 48 h after a series of washing operations. Then, it was pulverized by using a grinding device. The activation process of CA powder was carried out at a carbonization temperature of 600 °C for 2 h with heating rate of 5 °C/min, under N_2 atmosphere flow. The solid sample was left to cool down in the laboratory conditions. The samples were washed with dH_2O and dried in an oven at 105 °C.

Rumex patientia Extract Preparation

Rumex patientia extracts as a reducing agent were used in the preparation of RP-Ag/AC NPs. The preparation of *Rumex patientia* extracts was performed according to a procedure given in literature [28]. At first, the leaves of *Rumex patientia* (RP) were washed with deionized water and allowed to dry for 4 days at room temperature (RT). The dried leaves were crushed into powder. Next, 10 g of powdered leaves was mixed with 100 mL of deionized water and stirred at 80 °C for 45 min. The mixture was allowed to cool down to room temperature (RT) and the filtrate (extract) obtained by vacuum filtration was preserved in the oven.

Synthesis of RP-Ag/AC NPs

The synthesis of RP-Ag/AC NPs was carried out as given in literature [9] with a few minor changes. Briefly, a solution containing 10 mL of RP leaf extract and 40-mL solution of 0.02 g AgNO3/0.02 g AC particles was prepared. The prepared 50-mL solution was separated by centrifugation at 12,000 rpm for 20 min. The separated Ag/AC NPs were washed through a series of wash cycles to ensure that the Ag/AC NPs were free of biomaterials and dried in an oven at 75 °C for 2 h. Purified Ag/AC NPs were subjected to characterization analysis. The green synthesis mechanism process for RP-Ag/AC NPs is shown in Fig. 1.

Characterization of RP-Ag/AC NPs

XRD analyses of RP-Ag/AC NPs were performed with Panalytical EMPYREAN (Malvern Panalytical Ltd., Malvern, UK). TEM analyses were performed with Hitachi HT7800 (Hitachi Ltd., Tokyo, Japan). The average particle size was

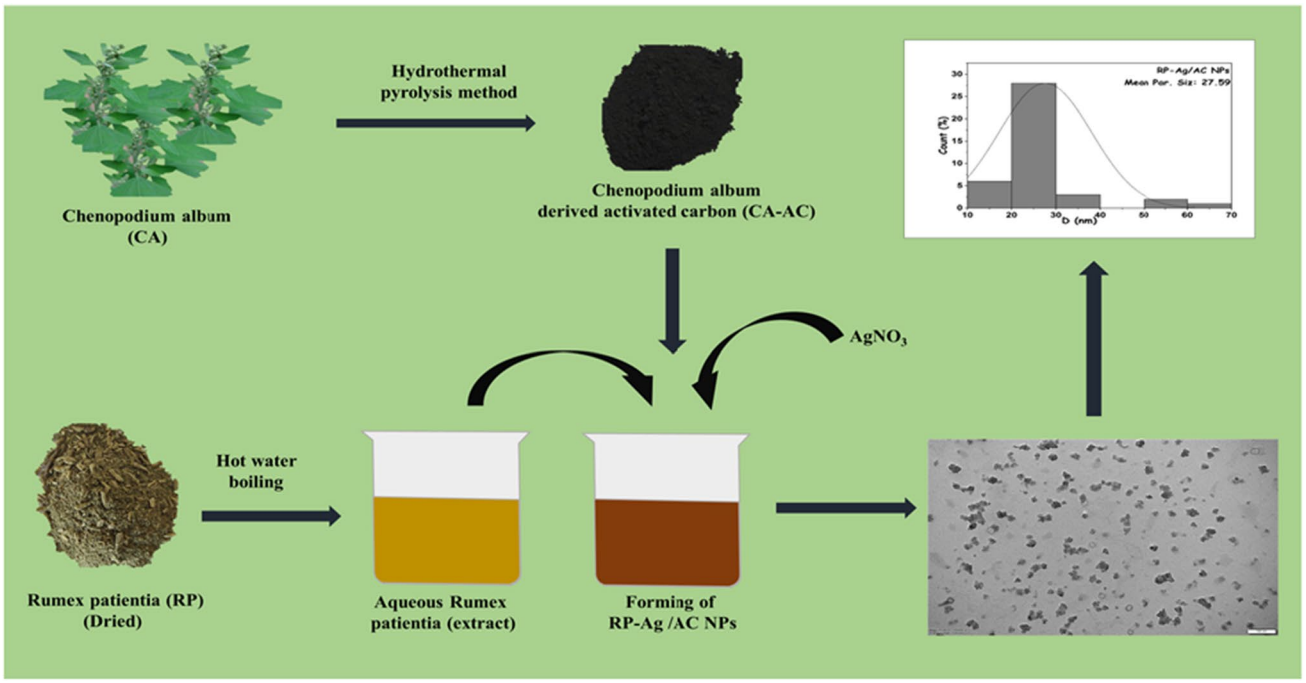

Fig. 1 Green synthesis of silver supported on activated carbon nanoparticles (RP-Ag/AC NPs) using the floral extract of *Rumex patientia* leaf extract as a reducing and stabilizing agent

calculated by measuring the spherical particles using TEM analysis images in the Imaging program as described previously [29].

In Vivo *Study*

Zebrafish

Adult zebrafish (*Danio rerio*) were raised at Izmir Biomedicine and Genome Center Zebrafish Facility and kept at 28 °C water temperature with a 14-h light/10-h dark cycle according to the standard procedures [30]. Fish were fed twice a day (9 a.m.–3 p.m.) with commercial feed (TetraMin Tropical Flakes, 48% protein, 8% fat, and 2% fiber) in the morning and *Artemia* in the afternoon. Zebrafish larvae used in this study were younger than 5 days post fertilization (dpf), eliminating the requirement of an ethics committee permission (Directive 86/609/EEC and EU Directive, 2010/63/EU).

Exposition of RP-Ag/AC NPs to Zebrafish Embryo

RP-Ag/AC NPs were applied to the zebrafish as follows: The stock solution of RP-Ag/AC NPs (2000 mg/L) was sonicated (50/60 kHz, HUBER Minichiller Diagenode, Germany) in ultrapure water for 3 h to prepare four different working 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 $CaCl_2$). Media were refreshed every 24 h after sonicating the solution [31, 32]. This procedure has been proved as an effective method to distribute and prevent agglomeration of nanoparticles in aquatic suspensions [33]. The study was planned based on a semi-static test with 4 replications [34]. The experiment was composed of five groups, including the control group with the embryos kept untreated in E3 medium. In total, 30 embryos were used for control and treatment groups. In the treatment groups, 5 mL of RP-Ag/AC NP solution at the defined concentration was added onto the embryos (exposed after 40 h of fertilization) and incubated for 96 h. All groups were kept at 28 °C and monitored daily.

Determination of Survival Rate, Hatching Rate, and Embryo Morphological Changes

The zebrafish (*Danio rerio*) embryos and larvae (in the control and treatment groups) were visualized using an SZX16 Olympus stereomicroscope (with an SC50 Olympus camera) between 24 and 96 h. When heartbeat was not observed in embryos at 24 hpf (hours post fertilization), the result was evaluated as death [26].

Determination of LC_{50}

LC_{50} value was calculated at 96 hpf by Probit analysis via Excel program [35]. For this purpose, fertilized *D. rerio* eggs

were exposed to RP-Ag/AC NPs, the dead embryos were detected after 96 h, and Probit analysis was performed. The data yielded an S-shaped curve [36]; thus, the LC value was calculated by converting doses into log10 to make this curve linear [37, 38].

Histopathological and Immunofluorescence Assays

The zebrafish larvae were incubated in 4% paraformaldehyde solution for 48 h. Following the routine tissue processing procedure, the larvae were embedded in paraffin blocks and sectioned at 4 µm. Histopathological sections were stained with the hematoxylin–eosin (HE) staining procedure and examined under light microscopy (Olympus BX 51, Germany). The data were evaluated as none (-), mild (+), moderate (++), and severe (+++) according to the degree of immunopositivity.

The sections taken onto poly-L-lysine slides were kept in dH_2O after deparaffinization and dehydration processes. They were boiled twice for 5 min to mask the antigens in the cells and allowed to cool down at room temperature (RT). The sections were incubated with the protein blocking solution (cat no:ab64226, Abcam), kept for 10 min at room temperature (RT). Primary antibodies nNOS and 8-OHdG (Cat No.: ab16650, diluent ratio: 1/100, sc-66036, Santa Cruz, EU) were applied according to the datasheet. After washing with PBS, sections were incubated with the secondary FITC antibody (FITC Cat No.: ab6785, diluent ratio: 1/1000) for 45 min in the dark at room temperature (RT) and washed in dH_2O. Sections are stained for DAPI in mounting medium (Abcam, ab104140, diluent ratio: 1/250) and kept in the dark for 5 min at room temperature (RT), washed with dH_2O, and covered with a coverslip. The sections were examined under a fluorescence microscope (Zeiss Leica DM 1000, Germany). The data were evaluated as none (-), mild (+), moderate (++), and severe (+++) according to the degree of immunopositivity.

Statistical Analyses

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

Results

Solid Material Characterization for RP-Ag/AC NPs

For XRD and TEM analyses, device settings were set as described previously [28, 39]. In XRD analysis (Fig. 2a), the prominent peaks at 26.76 were derived from the carbon element because of its crystal fringes (002). Peaks at 39.73°, 43.78°, and 47.58 corresponded to the crystal fringes (111, 200, and 220) of Ag element. As seen in TEM analysis of RP-Ag/AC NPs (Fig. 2b), Ag metals were immobilized on the AC surface. The cross dimensions of the structures in the clusters were calculated using the ImageJ and Origin programs as an average particle size of 27.59 nm (Fig. 2c).

Determination of LC_{50} Value at 96 hpf

The LC_{50} value was determined at 96 h in the zebrafish larvae that were exposed to different doses of RP-Ag/AC NPs starting the fertilization (0 hpf). The Probit analysis was conducted with the mean values by determining the live and dead embryos. The LC_{50} value at 96 h was found as 217.23 mg/L for RP-Ag/AC NPs.

Determination of the Effects of RP-Ag/AC NPs on the Larval Survival Rate, Hatching Rate, and Morphology

To determine the potential toxic effects of green synthesized RP-Ag/AC NPs, zebrafish embryos were exposed to the NPs at concentrations of 10, 50, and 100 mg/L. When the control and treatment groups were compared, survival rate was found to have decreased significantly in all groups in a dose-dependent manner in zebrafish embryos/larvae that were exposed to RP-Ag/AC NPs for 96 h (Fig. 3, $p < 0.05$). The difference between 50 and 100 mg/L groups was not significant (Fig. 3, $p > 0.05$). When the larval hatching rates were examined from 48 to 96 hpf, no significant difference was observed between the control and treatment groups of 10 and 50 mg/L at 72 and 96 hpf (Fig. 4, $p > 0.05$). However, larval hatching was found to have decreased significantly in both 50 mg/L (57%) and 100 mg/L (25%) groups compared to the control group at 48 hpf (Fig. 4, $p < 0.05$). Moreover, morphological defects such as yolk sac edema, pericardial edema, spinal curvature, and tail malformation were observed in all groups exposed to RP-Ag/AC NPs (Fig. 5). The highest rate of morphological abnormality was determined as pericardial

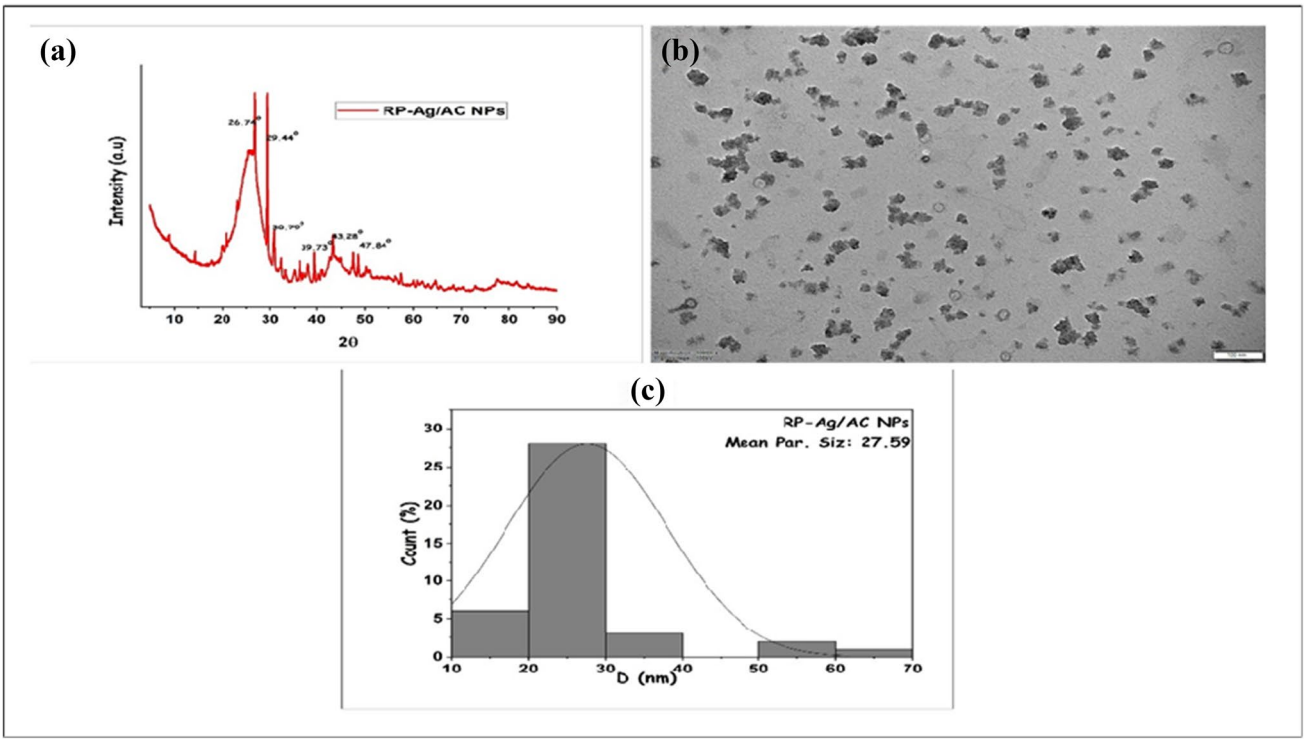

Fig. 2 The crystal particle size of RP-Ag/AC NPs and its mean crystal particle size

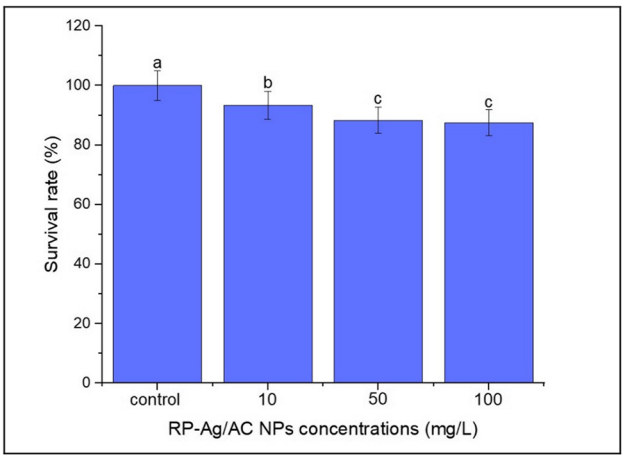

Fig. 3 The dose-dependent survival effects of RP-Ag/AC NPs on zebrafish embryo/larvae at 96 hpf. Data are expressed as means ± SD from three independent experiments ($p < 0.05$; ANOVA, Tukey's test). Different letters indicate significant differences between the groups ($p < 0.05$)

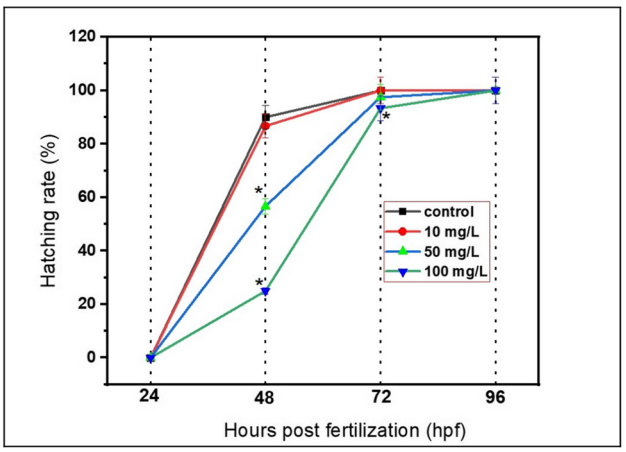

Fig. 4 The hatching rate of zebrafish embryos exposed green syntheses RP-Ag/AC NPs from 24 to 96 hpf. *$p < 0.05$

edema (19%) in the 100 mg/L treatment group (Fig. 5). All of these malformations were observed at significant rates between the control group and 50 and 100 mg/L groups (Fig. 5, $p < 0.05$).

RP-Ag/AC NPs Result in Oxidative Damage in Zebrafish Larvae

Especially in the investigation of neurological and neuropsychiatric disorders in humans, model organisms and important biomarkers (AcHE, ACH, nNOS or NOS-I, inducible NOS (iNOS or NOS-II), and endothelial NOS (eNOS or NOS-III)) are used. nNOS, Ca^{2+}-dependent, the central

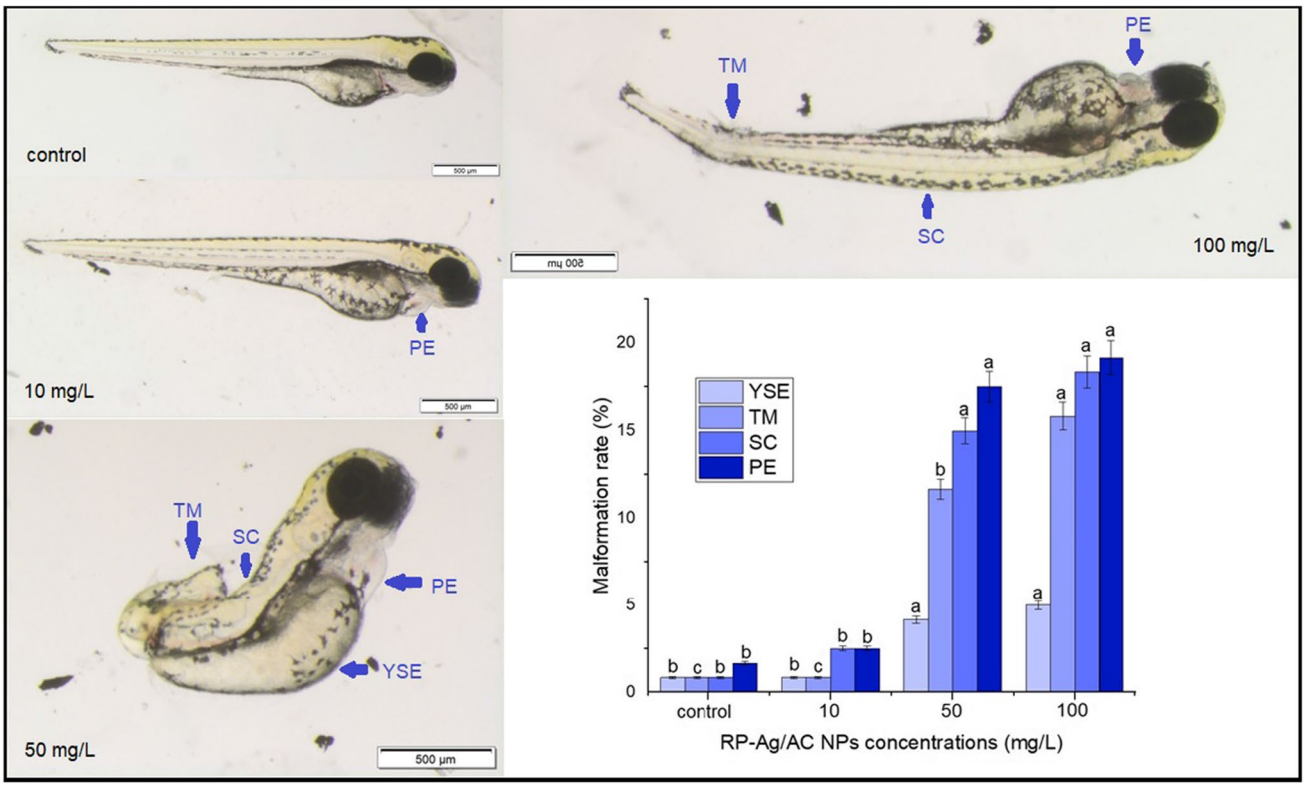

Fig. 5 Microscopic images of embryos and percentage of observed malformations after RP-Ag/AC NP exposure during 96 h. YSE, yolk sac edema; PE, pericardial edema; SC, spinal curvature; TM, tail malformation

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

	Control	10 mg/L	50 mg/L	100 mg/L
Degeneration in neutrophils	-	+	+ +	+ + +
Necrosis in neutrophils	-	-	+	+ +
8-OHdG	-	+	+ +	+ + +
nNOS	-	+	+ +	+ + +

nervous system functions not only by producing nitric oxide and peroxynitrite, but also by mediating several protein–protein interactions in neurons [40]. The most important and widely used biomarkers of oxidative damage in brain tissue are 8-OHdG and nNOS.

To test whether RP-Ag/AC NPs cause neutrophil-induced oxidative damage, first H&E staining was performed on larval tissue sections. In the 10 mg/L group, mild degeneration was detected in neutrophils (Table 1). In the 50 mg/L group, mild necrosis and moderate degeneration were detected in neutrophils. In the highest concentration (100 mg/L) group, moderate necrosis and severe degeneration were detected in neutrophils of zebrafish larvae (Table 1). Next, expression of the oxidative stress markers 8-OHdG and nNOS was examined by immunofluorescent staining of larval tissue sections. While there was no detectable expression of 8-OHdG and nNOS in the control group, in the 10 mg/L group, there was a mild 8-OHdG expression in neutrophils and mild nNOS expression in microglia and astrocytes (Fig. 6). In the 50 mg/L group, there was a moderate level of 8-OHdG expression in neutrophils and moderate nNOS expression in microglia and astrocytes. Finally, in the 100 mg/L group, there were high levels of 8-OHdG and nNOS in the relevant cells (Figs. 6 and 7).

Discussion

Plants, especially those called medicinal plants, contain important bioactive compounds. Recently, integration of medicinal plants with nanotechnology applications has demonstrated the therapeutic efficacy of herbal remedies in many diseases. With the development of biotechnology, the performed studies provide a lot of evidence about the secondary metabolites in plants which contain a wide variety of bioactive pharmaceutical components and their potential as therapeutic agents in the treatment of various diseases [16]. Identification of the active components of this plant and/or its extracts, which are widely used in traditional folk medicine, and determination of their therapeutic potential

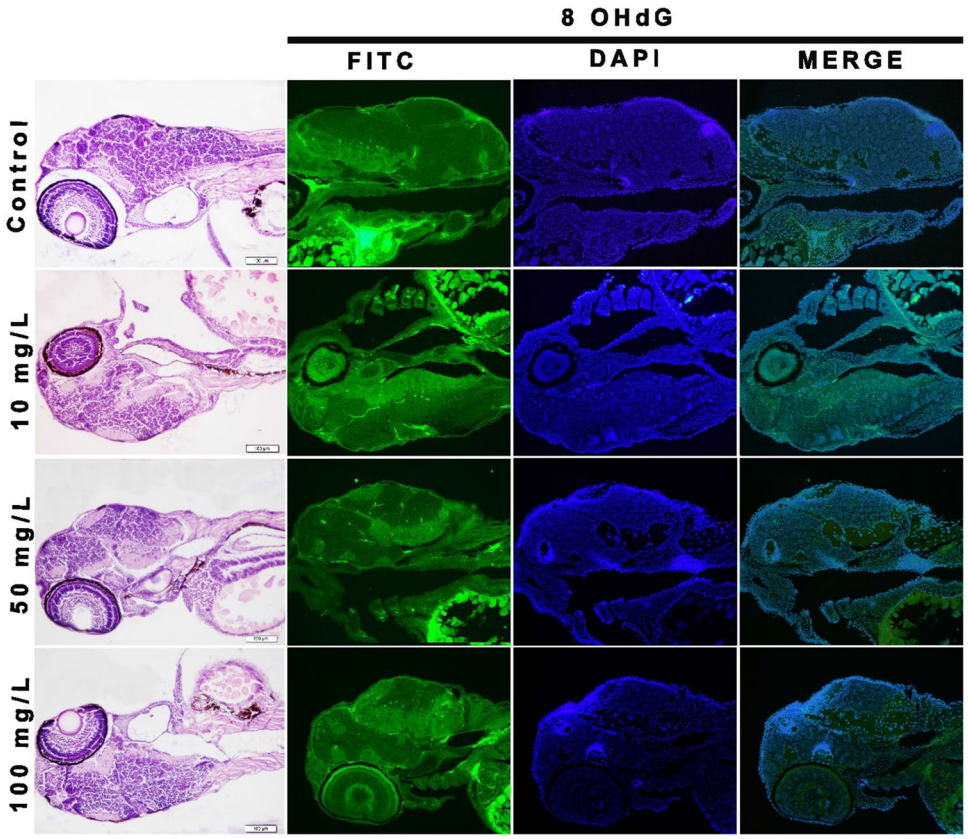

Fig. 6 Histopathological (H&E) and immunofluorescence (8-OHdG) images of zebrafish larvae samples, bar: 50 µm

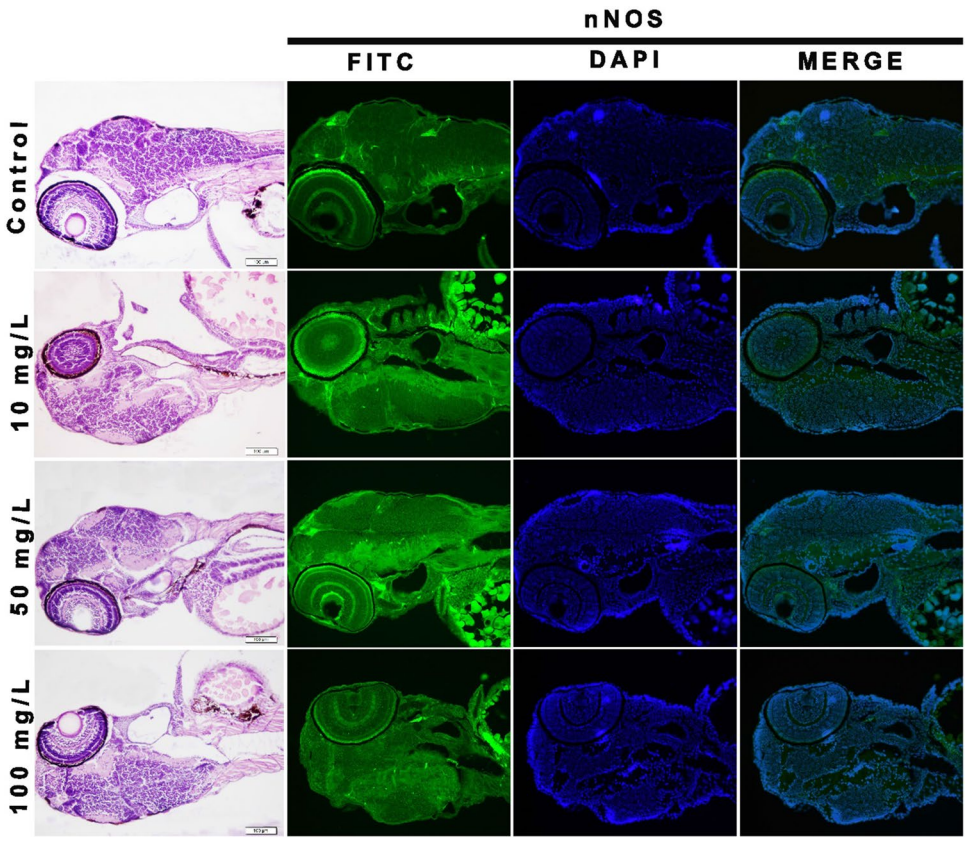

Fig. 7 Histopathological (H&E) and immunofluorescence (nNOS) images of zebrafish larvae samples, bar: 50 µm

support the ethno-pharmaceutical use of the plant. Previous studies concluded that plants show a number of therapeutic properties such as antioxidant, anticancer, antimicrobial, antiangiogenic, antifungal, anticholinesterase, hypoglycemic, and antiproliferative [12, 16]. In this study, the synthesized RP-Ag/AC NP's low efficacy is thought due to poor absorption and low compatibility and this situation was supported by Shehabeldine et al. [41] too. AgNPs exhibit different physicochemical properties depending on their size, shape, surface chemistry, temperature, pH, and ionic strength. These factors trigger different aggregation behaviors and precipitation or dissolution of AgNPs and have a significant impact on toxicity. Based on the results from previous studies, we assumed that the toxicity of AgNPs depends on the environment of the AgNPs [42]. The average size of the synthesized green nanoparticles (RP-Ag/AC NPs) was determined as 27.59 nm, which was found to be effective at the LC_{50} value (217.23 mg/L at 96 h). In a similar toxicity study for AgNPs, 12-nm NPs (LC_{50}: 50.1 µg/mL) were found to be more bioactive than 21-nm NPs (LC_{50}: 15.8 µg/mL) in zebrafish embryos [43]. Thus, although size-dependent toxicity is often observed in NPs' nanotoxicity [9, 44], other physicochemical parameters, experimental factors (i.e., endpoints, exposure conditions, test environment), and bioavailability of the particle can also affect toxicity [45, 46].

Embryotoxicity studies are often performed for biomedical applications on developing mammals. However, the zebrafish constitutes a useful pre-clinical alternative to mice for testing the effect of the drugs at nanoscale. Zebrafish are widely used to assess the toxicity of nanoparticles at embryonic, larval, and adult stages [47]. In the present research, malformations were detected in embryos exposed to RP-Ag/AC NPs. These findings were not compatible with those of Floris et al. [47], likely due to differences in nanoparticulate carriers, reactants, and concentrations. It is well known that NPs can overcome some of the mechanical barriers created by a biological system due to similarity to cellular proteins and components [48]. The differences in zebrafish larval phenotypes in these studies may be due to chemical residues from the synthetic treatment or to those that essentially "coat" the particles. Many reactants employed in NP synthesis may be toxic in vivo and in the environment above certain concentrations [48].

The molar concentration of the NPs accurately represents the size of the NPs, the number of moles, and surface properties. Therefore, the dose-dependent effects of NPs' molar concentrations on embryonic development show the dependence of nanotoxicity on the size, number, and surface properties of the NPs and hence accurately reflect the dose-dependent nanotoxicity [49]. Ninety-six hpf zebrafish embryos are cellular stages prior to tissue development. Ag^+ ion causes reactive oxygen species (ROS) generation, deoxyribonucleic acid (DNA) damage, or mitochondria destruction during development. Therefore, AgNPs can have a highly adverse effect on zebrafish embryos [42].

Khan et al. [50] had reported higher rates of malformation and mortality in green synthesis NPs (20–25 nm) in zebrafish. It is likely that the particle size we generated (27.59 nm) and the use of activated carbon reduce toxicity, probably by allowing less substance to penetrate into the cells.

AC is a non-toxic sorbent that is frequently used in the pharmaceutical and cosmetic industries to remove many toxins from the gastrointestinal system and stratum corneum. Thus, it can provide many therapeutic benefits due to its convenient modification using biotransformation processes in the gastrointestinal tract [51]. Powdered activated carbon has been used for water treatment to fight with the histopathological problems in fish liver, gills, and kidney [5]. The use of activated carbons increases the catalytic activity of many chemical and biochemical reactions [44]. Activated carbon has been shown to reduce toxic effects by interfering with the formation of certain chemical substances in zebrafish embryos [27, 52]. The present study further supports the beneficial use of activated carbon, one of the components of these green synthesis nanoparticles (RP-Ag/AC NPs), by reducing the Ag toxicity.

The active component and extracts of plants' research had showed that these substances can act by regulating the proliferation rate of immune cells at the cellular level in the organism, by affecting antibody production at the humoral level, or by increasing/decreasing the cytokine production and other mediators (phenolic compounds) [12].

Results show that RP-Ag/AC NPs cause oxidative DNA damage in zebrafish larvae. However, previous research on AgNPs has reported that they have higher genotoxic potential than Ag ions [45]. AgNPs caused more severe damage than Ag ions when apoptosis and oxidative stress were evaluated in HeLa cells and when the frequency of micronucleus formation was measured in HepG2 cells [39, 45, 53].

The findings of this study are consistent with those of Ahn et al. [45], where more DNA damage occurs after exposure to AgNPs likely due to generation of reactive oxygen species of AgNPs. Oxidative stress is known to cause permanent damage to the cell membranes, protein structures, and DNA lipids in the brain and other tissues. In recent years, many neurodegenerative diseases, especially in toxicity studies, have reported to cause inflammation, oxidative stress, and apoptosis in the brain tissue [19–21]. Dose-dependent degeneration and necrosis in neutrophils and nNOS expression in astrocytes and microglia cells due to RP-Ag/AC NPs are compatible with the recent study that has reported

a significant increase in the NOS activity after Ag-CS NC exposure in all tissues of bivalves [54].

Conclusion

In this study, high doses (50 and 100 mg/L) of RP-Ag/AC NPs were found to cause neurotoxicity, reduction in survival and hatching rates, and embryonic malformations in the zebrafish. The differences in altitude, temperature, active components of the plant *Rumex patientia*, the extraction technique, and the plant part used for extraction (leaf, root, stem) are thought to influence the toxicity of RP-Ag/AC NPs. Due to the toxicity, this plant extract should be used with carriers other than Ag for potential therapeutic purposes.

Considering that the discovery of new therapeutic agents against AgNP toxicity is a challenge, it can be said that *Rumex patientia* (RP) plant has a potential mitigating effect on the stress condition caused by low concentrations of AgNPs.

Overall, the results show that the coatings and particle size on the surface of RP-Ag/AC NPs contribute to the RP-Ag/AC NPs toxicity, and mitochondrial-DNA damage associated with oxidative stress appears to be a potential toxicity mechanism.

Based on the obtained data, it will be important to obtain more effective results in studies to be conducted to elucidate different toxicity pathways and determine therapeutic agent potentials. This study will provide information on the behavior of nanomaterials in environments, as well as tips on the use of different plant origin supplements to minimize the effects on organisms.

Author Contribution Mine Köktürk: the owner of the research idea and formal analysis. Serkan Yıldırım: resources and formal analysis. Mehmet Salih Nas: green synthesis. Gunes Ozhan: writing—review and editing. Ismail Bolat: resources and formal analysis. Mehmet Harbi Calimli: green synthesis. Muhammed Atamanalp: conceptualization, visualization, writing—review and editing. Gonca Alak: formal analysis, visualization, writing—original draft, writing—review and editing. All the authors read and approved the final version.

Availability of Data and Materials Not applicable.

Declarations

Ethics Approval The study does not require any license (Directive 86/609/EEC and EU Directive, 2010/63/EU).

Consent to Participate Fisheries, Chemistry, Environmental Science, Natural Science.

Consent to Publish *Biological Trace Element Research* has consent to publish this article.

Competing Interests The authors declare no competing interests.

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