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Cytotoxicity of silver nanoparticles obtained from *Eruca vesicaria* on the rainbow trout gonad cell line-2 (RTG-2)

Gökkuşağı alabalığı gonad hücre hattı-2 (RTG-2) üzerinde Eruca vesicaria'dan elde edilen gümüş nanoparçacıkların sitotoksisitesi

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Abstract
The rising applications of silver (Ag) nanoparticles in many sectors such as food, medicine, and agriculture lead to toxic effects on the ecological environment. Thus, studies on biological synthesis methods are carried out in order to diminish the toxicity caused by Ag nanoparticle synthesis methods. However, studies on the toxicity of biosynthesized Ag nanoparticles on fish cell lines are very few. The purpose of this research was to perform the biological synthesis of Ag nanoparticles via *Eruca vesicaria* plant extract and to examine their toxicity in the rainbow trout gonad cell line-2 (RTG 2). The characterization of Ag nanoparticles obtained from *E. vesicaria* was done by UV-vis, TEM, and XRD. The toxicity of Ag nanoparticles (100 µg/mL- 6,25 µg/mL) in the RTG-2 cell for 24 hours was determined by sulforhodamine B assay. Ag nanoparticles were obtained in the form of a sphere, triangle, cube, and sizes of 5-20 nm showed significant a toxic effect on RTG-2 fish cells depending on the dose at $p \leq 0,001$ levels. This study is important in terms of proving that Ag nanoparticles were obtained by biological synthesis have a toxic effect on fish cell lines and showing that there is a need for studies to reduce the release of Ag nanoparticles to the environment rather than synthesis methods.

Keywords: Biological synthesis, Cytotoxicity, *Eruca vesicaria*, RTG-2 cell line, Silver nanoparticle

Öz
Gümüş (Ag) nanoparçacıklarının gıda, ilaç, tarım gibi birçok sektörde artan kullanımı ekolojik çevre üzerinde toksik etkilere yol açmaktadır. Bu nedenle Ag nanoparçacık sentez yöntemlerinin neden olduğu toksisiteyi azaltmak için biyolojik sentez yöntemleri üzerinde çalışmalar yapılmaktadır. Bununla birlikte, balık hücre hatlarında biyosentezlenmiş Ag nanoparçacıklarının toksisitesi üzerine yapılan çalışmalar çok azdır. Bu çalışmanın amacı, Eruca vesicaria bitki özü ile Ag nanoparçacıklarının biyolojik sentezini gerçekleştirmek ve gökkuşağı alabalığı gonad hücre hattı-2'de (RTG 2) toksisitelerini incelemektir. E. vesicaria'dan elde edilen Ag nanoparçacıklarının karakterizasyonu UV-vis, TEM ve XRD ile yapılmıştır. Ag nanoparçacıklarının (100 µg/mL- 6,25 µg/mL) 24 saat boyunca RTG-2 hücresindeki toksisitesi sülforodamin B tahlili ile belirlendi. Küre, üçgen, küp ve 5-20 nm boyutlarında elde edilen Ag nanopartiküller, p≤0,001 seviyelerinde doza bağlı olarak RTG-2 balık hücreleri üzerinde önemli toksik etki göstermiştir. Bu çalışma, biyolojik sentez yoluyla elde edilen Ag nanoparçacıklarının balık hücre hatları üzerinde toksik etkisinin olduğunun kanıtlanması ve sentez yöntemlerinden ziyade Ag nanoparçacıklarının çevreye salınımını azaltacak çalışmalara ihtiyaç olduğunu göstermesi açısından önemlidir.

***Anahtar kelimeler**: Biyolojik sentez, Sitotoksisite, Eruca vesicaria, RTG-2 hücre hattı, Gümüş nanoparçacık*

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1. Introduction

1. Giriş

Nanoparticles, which are called solid particles with a dimension range of 1-100 nm, exhibit improved properties including high biologic and chemical activity compared to bulk materials with the effect of their large surface area/volume proportion and quantum attraction forces (Tao et al. 2021). Therefore, studies on the use of nanoparticles are carried out in almost all areas where bulk materials are used, such as health, energy, communication, food, agriculture, chemistry, space technologies, cosmetic products, construction (Srivastava et al. 2021).

Silver (Ag) nanoparticles are one of the most studied metal nanoparticles. Because of the strong antimicrobial action of Ag nanoparticles, their use stands out especially in areas where hygiene and sanitation are very important such as health, medicine, sensing and imaging practices in food and biological compounds (Aritonang et al. 2019; Han et al. 2020; Velsankar et al. 2020). Global, the total guessed manufacture of Ag nanoparticles was almost 500 tonnes per year in 2009, and waiting for an increment of almost 900 tonnes by 2025 (Du et al. 2018; Islam et al. 2021). Physical, chemical and biological synthesis methods such as thermal separation, chemical reduction, electron irradiation, reduction in the presence of biological elements, laser ablation, self-assembly, photochemical reduction, microwave-assisted synthesis have been reported to obtain Ag. The existence of potential ecological and biological risks arising from the usage of poisonous chemical reagents in chemical and physical synthesis routes has increased the demand for biological synthesis routes nanoparticles, especially plant-based synthesis routes (Leon-silva et al. 2016; de Jesús Ruíz-Baltazar et al. 2018; Shahriary et al. 2018; Lee & Jun, 2019; Fırat Baran, 2019; Umaz et al. 2019; Ulaeto et al., 2020; Hatipoğlu, 2021). There are a large number of researches on the biological synthesis of Ag nanoparticles via the plant media (Dura et al. 2019; Karahan & Çölgeçen, 2021). However, a study on the synthesis of Ag nanoparticles via *Eruca vesicaria* has not been reported.

There are reports that biological synthesis pathways have less environmental toxicity due to the absence of synthetic chemicals compared to other methods (Bélteky et al. 2019; Gamboa et al. 2019). Aquatic organisms, which are one of the best indicators of environmental contamination, are very suitable for investigating nanoparticle toxicity (Demir et al. 2020). Although there are many studies on the antimicrobial effects of Ag nanoparticles obtained by plant-based biological synthesis, there are few studies examining its ecotoxicological effects on aquatic organisms. *In vitro* studies are ideal tools for assessing chemical toxicity at the cellular level prior to *in vivo* studies (Bermejo-Nogales et al. 2017). The rainbow trout gonad cells (RTG-2) have been used widely as a standard fish cell line for toxicity assays and determined to be more responsive than standard mammalian cells (Vevers & Jha, 2008).

In this study, the aims were: 1) the biological synthesis of Ag nanoparticles via *Eruca vesicaria* and 2) the determine the cytotoxicity of Ag nanoparticles at different concentration on the RTG-2 cells using cell viability test.

2. Material and method

2. Materyal ve metot

2.1. Preparation of *Eruca vesicaria* extract

2.1. Eruca vesicaria ekstraktının hazırlanması

Eruca vesicaria plants were obtained from a local green grocery store located Erzurum, Turkey. 25 g of *E. vesicaria* plant leaves were weighed. Next, it was washed 2-3 times with tap water and distilled water at least two times to remove pollution such as sludge, dust, insect waste. After leaves were cut into small pieces, it was boiled with 200 mL distilled water at 75 °C for 15 min (boil for 5 min) and cooled at room temperature. The plant extract was filtered with Whatman filter paper No. 1 (Baran et al. 2022). Then, it was stored at refrigerator temperature (4 °C) for Ag nanoparticle synthesis.

2.2. Biological synthesis of silver nanoparticles

2.2. Gümüş nanoparçacıkların biyolojik sentezi

500 mL, 1 mM solution of $AgNO_3$ (Sigma-Aldrich Cas No: 7761-88-8) was prepared. 10 mL of *E. vesicaria* extract was mixed this solution and then, this mixture was kept in a dark closet because of avoid photo-activation of silver nitrate at room temperature. 1-2 min after the plant extract was added, the colorless $AgNO_3$ solution turned brown-red. This colour change confirmed the reduction from Ag^+ to Ag^0. Then, centrifugation and washing were carried out at least 3 times in order to remove from the plant residues, respectively, and finally the product was dried in an oven (Memmert UN110) at 75 °C (Baran et al. 2021).

2.3. Physical and chemical characterization of silver nanoparticles

2.3. Gümüş nanoparçacıkların fiziksel ve kimyasal karakterizasyonu

Characterization of the silver nanoparticles was performed using UV-vis spectral analysis, Transmission electron microscopy (TEM), and X-ray diffractometer (XRD). These analyses were done by using Shimadzu UV-visible spectrophotometer (UV-3600 Plus), Hitachi Ht7700 microscope, and PANalytical Empyrean X-ray diffractometer in DAYTAM, Atatürk University, respectively. The particle size and surface morphology of silver nanoparticles was analysed using TEM, operated at an accelerated voltage of 120 kV after sonicating ones for 1 h in ultrapure water. UV–vis spectrometer was used to record absorbance in the range of 200–1100 nm. Crystal structure of silver nanoparticles was examined by X-ray diffraction analysis with a Co-Kα1 radiation in the range of 10 to 90° (Behravan et al. 2019; Baran et al. 2021).

2.4. Growth of the rainbow trout gonadal cells (RTG-2) and treatment of silver nanoparticle

2.4. Rainbow trout gonad hücrelerinin (RTG-2) büyütülmesi ve gümüş nanoparçacık uygulaması

The RTG-2 cell line was obtained from Türkiye Şap Enstitüsü, (Ankara, TURKEY). The RTG-2 cell line (passage 12-15 used for experiment) was cultured in Eagle's minimal essential medium (EMEM: with L-glutamin medium, ATCC 30-2003) supplemented with %10 fetal bovine serum (Biowest S1810-500) and %1 penicillin-streptomysin (Sigma P4333) at 22±2 ˚C. Cell cultures were kept in 25 cm^2 culture flasks (Isolab 120.11.025) and the medium was changed every 2 days. When cells covered 80% of the flask, they were removed using 0.5% trypsin/0.02% EDTA (Gibco 25200072). Then, cells were seeded into 96-well plates (Isolab 122.11.096) at $3x10^4$ cells per well (final volume 130 µL medium) and incubated for 24 hours.

Ag nanoparticles at different concentrations (100 µg/mL- 6,25 µg/mL) were dissolved in the medium with the help of an ultrasonicator and applied to cells planted 24 hours ago on 96-well plates (n=6). Dimethylsulfoxide (DMSO Sigma Cas No: 67-68-5) was used as a positive control. After 24 hours of incubation, cell viability test was performed with sulforhodamine B (SRB Sigma Cas No: 3520-42-1) assay.

2.5. Cell viability assay

2.5. Hücre canlılık testi

Developed in 1985 to measure cell proliferation and cytotoxicity, the SRB assay is a colorimetric method based on the in vitro measurement of the total protein content of cells. 24 hours after the application, 100 µl of 10% cold trichloroacetic acid (TCA Isolab CAS No:76-03-9) was added to each well and fixed at +4 ºC for 1.5 hours. Then, TCA was removed from the plate (plate is poured by inverting). The wells were washed 5 times with deionized water to remove TCA. At the end of each wash, the plate was turned upside down and poured. Then, 50 µl of the SRB solution (0.04% wt/vol) was added to each well and kept at room temperature in the dark closet during 30 min. Then, the SRB was poured off the plate and the wells were washed 5 times with 1% acetic acid (Sigma Cas No: 64-19-7) to remove unbound dye, and the wells were dried in the cabinet without any drops. In order to dissolve the dye bound to the proteins, 10 mM tris base (150 µl/well) (Sigma 901.013.2500) was added and the plate was kept on a shaker for at least 10 min to homogenize the dye solution. (approximately 150rpm). Optical density was read at 564 nm on an ELISA reader (Biotek Epoch) (Vichai & Kirtikara 2006; Orellana & Kasinski 2016). The viability of untreated control cells was accepted as 100%, and the viability rates of the treated cells were calculated using the formula (1) below:

% Viability=[100×(Compound-treated cell absorbance mean-blind mean)/(Control cell absorbance mean-blind mean)] (1)

2.6. Statistical analysis

2.6. İstatistiksel analizler

Statistical evaluation of SRB test results was performed by Tukey pairwise comparison method of one-way ANOVA test. The difference between the results with a significance value less than 0.05 ($p<0.05$) was considered significant. SPSS 20 (IBM, USA) software was used for all statistical analyses (Sezgin et al. 2019).

3. Results

3. Bulgular

3.1. Biological Synthesis of Ag nanoparticles

3.1. Gümüş nanoparçacıkların biyolojik sentezi

The biological synthesis of Ag nanoparticles was confirmed by macroscopic observation, with the reaction colour turning brown-red (Figure 1).

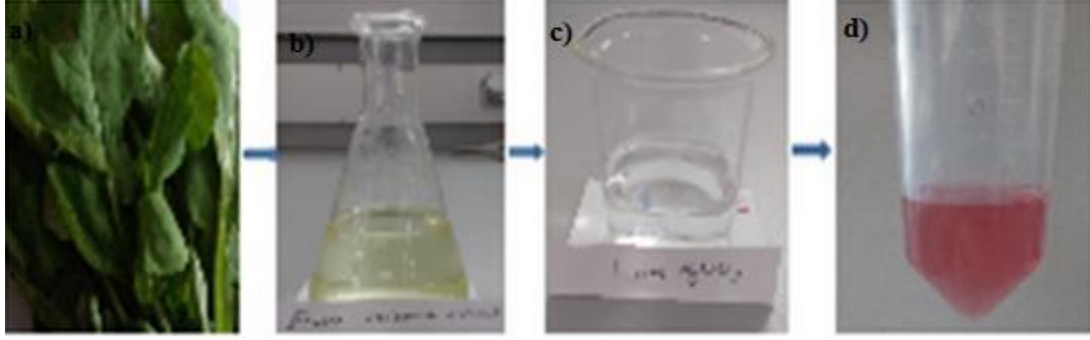

***Figure 1**. The biological synthesis of Ag nanoparticles a) E. vesicaria plant b) E. vesicaria plant extract c) 1 mM solution of $AgNO_3$ d) Color change indicating the nanoparticle synthesis*
Şekil 1. Ag nanoparçacıkların biyolojik sentezi a) *E. vesicaria* bitkisi b) *E. vesicaria* bitki ekstraktı c) 1 mM $AgNO_3$ solüsyonu d) Nanopartikül sentezine işaret eden renk değişimi

3.2. Physical and chemical characterization of silver nanoparticles

3.2. Gümüş nanoparçacıkların fiziksel ve kimyasal karakterizasyonu

UV-Vis spectra of Ag nanoparticles aqueous is given in Figure 2. The peaks of silver nanoparticles were found between 250 nm and 450 nm. Purely ionic silver may show a contribution in the near UV range, around 300 nm wavelength. Petit et al. (2021) reported that the emission at 290 nm, a broad excitation band centered at 230 nm was observed, attributed to Ag+ ion. (Petit et al. 2021)

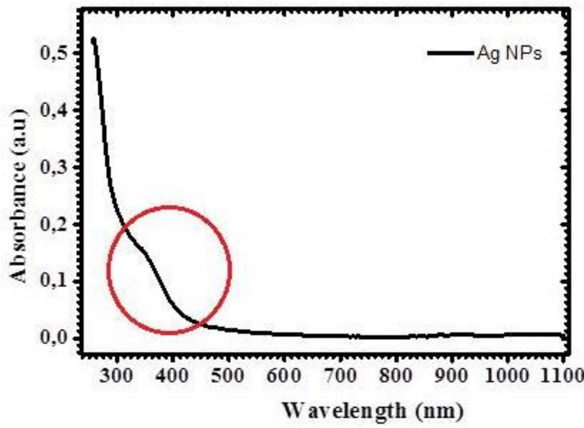

***Figure 2.** UV-vis spectrum of Ag nanoparticles*
Şekil 2. Ag nanoparçacıkların UV-vis spektrumu

The TEM image of silver nanoparticles is given in Figure 3. Silver nanoparticles have diameter ranging from 5 to 20 nm and a few 20-40 nm. This figure shows individual silver particles as well as a few aggregates. The morphology of the silver nanoparticles was observed as spherical, triangular and cubic nanoparticles according to the TEM image. The large particles in the TEM image look different from the spherical shape. However, it should be taken into account that large particles were actually spherical, and particles overlapping on the grid may be caused misunderstanding in terms of shape.

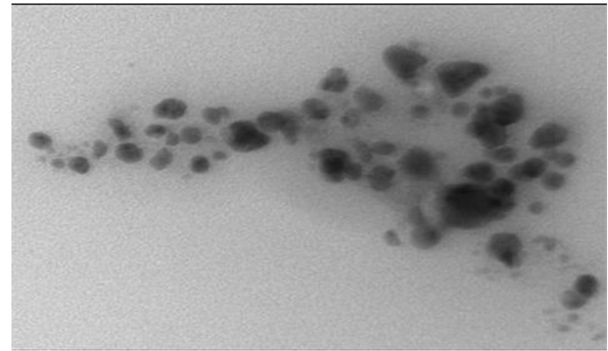

***Figure 3.** TEM image of Ag nanoparticles*
Şekil 3. Ag nanoparçacıkların TEM görüntüsü

X-ray diffraction pattern for silver nanoparticles represented Figure 4. The prominent peaks at 2θ = 38.08°, 41.18°, 46.25°, 64.53°, and 76.76° represents the (1 1 1), (2 0 0), (2 0 0), (220) and (311), respectively.

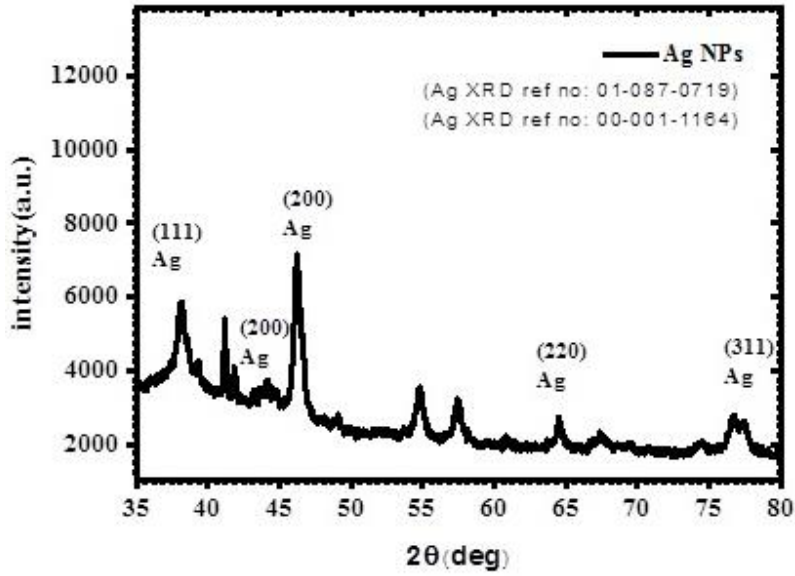

Figure 4. *XRD spectrum of Ag nanoparticles*
Şekil 4. Ag nanoparçacıkların XRD spektrumu

3.3. Cytotoxicity of silver nanoparticles on the RTG-2 fish cells

3.3. RTG-2 balık hücreleri üzerinde gümüş nanoparçacıkların sitotoksisitesi

Cytotoxicity of silver nanoparticles on the RTG-2 fish cells is given in Figure 5. The concentration (IC_{50} value) at which Ag nanoparticles killed 50% of the population compared to the control group was determined as 25 µg/ml. When the control group was accepted as 100% alive, the viability rate in this group was determined as 51.1%. Compared to the control group, all doses of Ag nanoparticle applied (100 µg/mL- 6,25 µg/mL) significantly reduced the viability of the RTG-2 fish cells. Compared to DMSO used as a positive control, 100 µg/mL and 50 µg/mL Ag nanoparticle applications have higher toxic effects. The toxicity of Ag nanoparticles was concentration dependent.

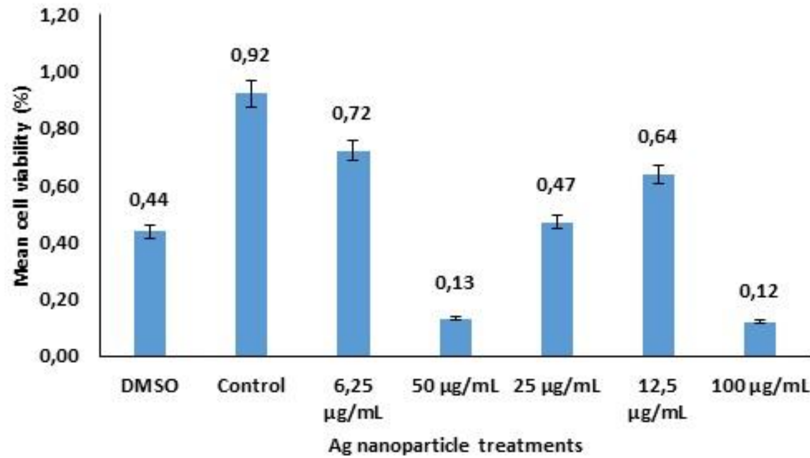

Figure 5. *Cytotoxicity of Ag nanoparticles on the RTG-2 fish cells*
Şekil 5. Ag nanoparçacıkların RTG-2 balık hücreleri üzerine sitotoksisitesi

4. Discussion

4. Tartışma

At the stage of synthesis of Ag nanoparticles, while the colour of the $AgNO_3$ solution was white and the colour of the plant extract was yellowish green, a reddish colour that gradually darkened over time was observed in the mixture of the two solutions. This is in accordance with the results obtained in other Ag nanoparticles biological synthesis researches (Abbas et al. 2017; Balachandar et al. 2019). Here, it is likely that phytochemicals of *E. vesicaria* such as alkaloids, alcohols, phenols,

saponins, terpenes and proteins play a role and reduce from Ag^+ to Ag^0 (Okaiyeto et al. 2021).

According to the UV visible spectrophotometer measurement, a high absorbance peak at approximately 250 nm gradually decreased, although it showed a very slight upward trend after approximately 360 nm, but it decreased (Figure 2). Ag nanoparticles showed absorbance values around 443 nm and 323 nm, respectively, in other studies using *Bidens Frondosa* and *Salvia officinalis* extracts (Abbas et al. 2017; Okaiyeto et al. 2021). Also, it has been reported in other studies that spherical shaped Ag nanoparticles show absorption peaks between 380 nm and 450 nm (De Aragão et al. 2016; Nogueira et al. 2019). Ag nanoparticles in spherical form observed in the TEM image (Figure 3) can explain the absorbance at wavelengths before 450 nm in the UV-vis spectrum. In the TEM image, it is clearly seen that Ag nanoparticles with an average size of 20 nm are in spherical, triangular and cubic shapes. The variable size of the nanoparticles may be due to the binding of protein and other molecules in the plant extract to the nanoparticle surface during the reduction of the nanoparticles. The size and shape forms obtained in this study are in accordance with the size and shape forms obtained in previous studies in which Ag nanoparticles were synthesized via plant extract (Okaiyeto et al. 2019; Alyousef et al. 2019; Gomathi et al. 2021). X-ray diffraction analysis was given in Figure 4 revealed the original Bragg reflection values of Ag nanoparticles; 38.08°, 41.18°, 46.25°, 64.53°, and 76.76° at 2θ angle, which complied with 111, 200, 200, 220 and 311, demonstrating the face-centered cubic (FCC) crystalline structure of the Ag nanoparticles synthesized using extract of *E. vesicaria* as the reducing factor (Baghizadeh et al 2015). The intense peak at 46.25° indicates a big grade of crystallinity. The high peaks at 44.50° and about 55.00° in the XRD spectra could be because of the presence of phytochemical and organic compounds in extract of *E. vesicaria* coating the surface of the synthesized Ag nanoparticles (Carmona et al. 2017; Kamaraj et al. 2017; Kora et al. 2020; Okaiyeto et al. 2021).

In vivo approaches are at the whole organism level, while *in vitro* studies (especially carried out in 2D) are at the cellular level and remain limited. Therefore, *in vitro* systems show less sensitivity when compared to *in vivo* systems. It was reported that LC_{50} value in the juvenile rainbow trout was 3.13 µg/mL and IC_{50} values ranged from 10.7 to 75.9 µg/mL in the rainbow trout cell lines (RTL-W1, RTH-149, RTG-2) following Ag nanoparticle (it obtained by chemical synthesis) treatment for 48 h (Johari et al. 2013; Connolly et al. 2015). The RTG-2 cell culture is one of the fish cell cultures that stand out especially in toxicity studies, as they have a high genetic similarity index with *in vivo* rainbow trout (*Oncorhynchus mykiss*), do not require an exogenous metabolic system, and maintain their ability to metabolize toxic substances (Caminada et al. 2006). In this study, the lowest concentration of 6.25 µg/mL and subsequent higher concentrations of 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL of Ag nanoparticles showed a significant toxic effects compared to the control group in the RTG-2 cells. Also, 50 µg/mL, and 100 µg/mL of Ag nanoparticles showed a significant toxic effects compared to positive control treatment. Dissolution of Ag nanoparticles applied in this study in a medium containing 10% fetal bovine serum may also have contributed to these differences in IC_{50} values arising from the differences between *in vitro* and *in vivo* systems. The concentrations of Ag nanoparticles that affect the cells in the culture medium containing fetal bovine serum may be lower due to the presence of serum proteins. Ag nanoparticles dissolved in bovine serum albumin-containing medium showed an effect at 31 µg/mL concentration, while Ag nanoparticles dissolved in serum free medium showed effects with 2.5 and 4.9 µg/mL concentrations in the rainbow trout primary hepatocytes (Farkas et al. 2010; Massarsky et al. 2014). In this study, fetal bovine serum was used to mimic the circulatory environment that nanoparticles are exposed to before they come into contact with gonad cells. Therefore, the usability of the RTG-2 cell line for cytotoxicity tests and its suitability for preliminary trials are obvious. In addition, the view that biological synthesis of silver nanoparticles reduces toxicity to organisms in the ecosystem, compared to chemical synthesis, is weakening. Consequently, there is a need for different approaches to reduce or prevent the release of Ag nanoparticles to the environment, especially living in aquatic ecosystems.

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Author contribution
Yazar katkısı

Semra ÇİÇEK: Research design, experimentation, analysis of data, writing of the article and approval of the final version.

Declaration of ethical code
Etik beyanı

The author of this article declares that the materials and methods used in this study do not require ethical committee approval and/or legal-specific permission.

Conflicts of interest
Çıkar çatışması beyanı

The author declares that there is no conflict of interest.

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