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A comparative study on aquatic toxicity of chemically-synthesized and green synthesis silver nanoparticles on *daphnia magna*

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ABSTRACT

The steady increase in the employment of silver nanoparticles (AgNPs) in consumer products entails the determination of the aquatic toxicity of AgNPs. Various AgNP characteristics including particle size, and shape, surface charge, and material have prominent effects on ecotoxicity. In the present study, we investigated the aquatic toxicity of chemically-synthesized AgNPs (Che-AgNPs) and green synthesis AgNPs (Gr-AgNPs) to *Daphnia magna* as a model organism. In each case, Che-AgNPs and Gr-AgNPs showed dose-dependent toxicity in the range of 5–50 ppb. It was also detected that the size and surface coverage material of AgNPs has a significant impact on the survival rate of *D. magna*. We also analyzed the expression of some genes related to detoxification and the reproductive system. These observations presented that in both NP types the significant alterations were detected in genes of the model organism in a dose-dependent manner.

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Acute toxicology; green synthesis; silver nanoparticles

Introduction

The employment of commercially available nanoparticle (NP)-based products has increased in the last years (Asghari et al. 2012). The exposure of nanoparticles by humans and the environment is inevitable and their potential effects are still unclear (Handy et al. 2008; Bystrzejewska-Piotrowska et al. 2009; McTeer et al. 2014). Particularly, silver NPs (AgNPs) are widely used in many consumer products including textiles, cosmetics, and medicals (Rani and Rajasekharreddy 2011). Since AgNPs are the most widely employed commercially products because of their antibacterial activities, their potential risk on the consumer and the environment is the highest in comparison to other NP systems (Hansen et al. 2008). The release of AgNPs from nanoparticle-based products into the environment leads to crucial contamination in almost all components of aquatic ecosystems such as organisms, communities, and environment (Geranio et al. 2009; Lorenz et al. 2012; Pasricha et al. 2012; Künniger et al. 2014; Krzyżewska et al. 2016). AgNPs represent different characteristics from their homolog bulk counterparts and therefore their eco-toxicity is mainly unique depending on the many parameters including particle size and shape, surface charge, and material.

Daphnia magna is a freshwater crustacean and among the most sensitive model organisms employed in eco-toxicity tests. The standard protocols proposed by International Standards

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Organization (ISO), Environmental Protection Agency (EPA), and Organization for Economic Cooperation and Development (OECD) dictate the usage of *D. magna* as a model organism (Asghari, Johari, Lee, Kim, Jeon, Choi, Moon and Yu 2012). In the aquatic ecosystem, *D. magna* plays an important role as a primary consumer of the food chain. Therefore, any significant alteration in their population size can be a good indicator of aquatic toxicology.

In literature, there have been many studies on the aquatic toxicity of AgNPs with different preparation techniques, stabilities, surface charge, and size in model aquatic organisms such as *D. magna* (Asghari, Johari, Lee, Kim, Jeon, Choi, Moon and Yu 2012; Griffitt et al. 2008; Allen et al. 2010; Kennedy et al. 2010; Gaiser et al. 2011; Sakka et al. 2016). Allen et al. reported the toxicity of silver nitrate, commercially available AgNPs, and laboratory-produced AgNPs (Allen et al. 2010). For all systems, they detected dose-dependent toxicity in the range of ppb scale, and the differences in toxicity for NP systems were attributed to the NP size. Similar results were also observed in the report by Asghari et al. (2012) (Asghari, Johari, Lee, Kim, Jeon, Choi, Moon and Yu 2012). They showed that the toxicity of AgNPs in *D. magna* was highly dependent on their chemical characteristics and aggregation level. Similarly, Blinova et al. investigated the toxicity of silver ions and AgNPs that were prepared by different strategies and stabilized with different agents (Blinova et al. 2013). With their *D. magna* toxicity tests, they concluded that silver ions represented higher toxicity than any type of AgNPs. In addition to chemically-synthesized AgNPs (Che-AgNPs), the role of green synthesis AgNPs (Gr-AgNPs) on aquatic organisms must be considered to determine their potential effects on the environment. Green synthesis procedures offer economic and efficient alternatives in an eco-friendly manner. Also, these procedures reduce the contamination of the water sources and environment and also minimize the employment of the toxic substances. In spite of the many approches to prepare the Gr-AgNPs, the reports on their effect on aquatic organisms are still limited. For instance, Rani et al. compared the toxicity of Gr-AgNPs and Che-AgNPs in *D. magna* as model organisms (Rani and Rajasekharreddy 2011). They used leaf extract of *Piper betle* in the synthesis of Che-AgNPs. They observed that in comparison to Che-AgNPs, the protein shell around the nanoparticle in the Gr-AgNP system reduced its toxicity remarkably.

Although there have been many reports on the aquatic toxicity of AgNPs in aquatic organisms, further studies are still required to determine their toxicity levels, mechanism, and comparison between nanoparticles prepared through different approaches. Herein, we evaluated the toxicity of Gr-AgNPs and Che-AgNPs in *D. magna*. We employed the extract of *Salvia aethiopis* L. plant leaves in the synthesis of Gr-AgNPs. In addition to toxicity tests, the expression of some genes related to detoxification (*cyp360a8, gst*, and *hr96*) and reproductive system (*cyp314*, and *vtg*) was performed to find out probable mechanism behind the toxicity, as many environmental pollutants can change the expression of the detoxification and antioxidant-related genes such as *cyp's* and *gst*. In this study, *hr96, cyp314, gst*, and *vtg* have been selected as target genes since *hr96* can regulate the expression of phase I, II, and III detoxification genes, cyp314 plays a role in the biosynthesis of ecdysone hormone, gst protects cells against reactive oxygen species, and vitellogenin, the precursor of the egg yolk protein, is a biomarker frequently used for exposure to estrogenic compounds. To the best of our knowledge, this is the first comparative report showing the differences in toxicity of Che-AgNPs and Gr-AgNPs in *D. magna* in terms of survival rate and gene expression level.

Materials and methods

Synthesis of NPs and characterization

In this study, we aimed to determine the toxicity of different NP systems including citrate stabilized-AgNPs (Che-AgNPs), and green synthesis AgNPs (Gr-AgNPs) in *D. magna*. For this, firstly, AgNPs were prepared according to the well-known citrate reduction method with some modifications similar to our earlier reports (Yilmaz 2019). Briefly, 25 mL of 1 mM silver nitrate was heated to boiling. Then, under vigorous magnetic stirring (1400 rpm), 2.5 mL of 1% tri-

sodium citrate solution was rapidly added to this solution. The synthesis of Che-AgNPs was confirmed through the emergence of the pale yellow color of the suspension. After complete reaction in 30 min, the heater was turned off and the suspension was cooled to room temperature. For the purification, the Che-AgNP system was centrifuged at 15,000 rpm for 20 min. The supernatant was discarded and the aggregated pellet was redispersed in DI water and stored at 4 °C in the dark until the next usage.

In the synthesis of the Gr-AgNP system, we employed the extract of *Salvia aethiopis* L. plant leaves collected from Erzurum, Turkey. Firstly, the plant leaves were washed with consecutive tap and DI water several times. Then, the leaves were dried at ambient conditions and grounded for the formation of fine pieces. For the extraction, 5 g of the grounded leaves were dispersed in 100 mL of DI water and heated to boiling for 1 h. The resultant extract was filtered through Whatman filter paper. The extract was stored in the dark at 4 °C. In Gr-AgNP synthesis, 100 mL of 10 mM $AgNO_3$ solution was mixed with 100 mL of the plant extract and stirred gently in a shaker at 24 °C. The synthesis of silver nanoparticles was monitored by using a UV-vis absorption spectrophotometer. After completion of the reaction in 3 h, the NP suspension was centrifuged at 15,000 rpm for 20 min and redispersed in DI water, and stored at 4 °C in the dark.

The plasmonic and morphological analyses of both Che-AgNPs and Gr-AgNPs were performed in detail through UV-vis absorption spectra and transmission electron microscopy (TEM), respectively.

Daphnia magna

D. magna was cultured and maintained similar to our earlier reports (Icoglu Aksakal 2019). Briefly, *D. magna* was cultured at given conditions: 21 ± 1 °C, 16:8 light: dark photoperiod, pH: 8.1 ± 0.2, and dissolved oxygen >6.2 mg/L. During the culturing period, *D. magna* was daily fed with *Scedenesmus obliques*.

Acute toxicity tests

Third and fourth brood neonates (aged < 24 h) were used in acute toxicity tests which were performed according to OECD guideline 202 (No 2004). For each run, 30 neonates were placed into a 500-ml-glass beaker containing 400 mL of NP suspension at the desired concentration (5–50 ppb). Four replicates were performed for each NP application and the neonates were exposed to NPs for 48 h. Afterward, the neonates have transferred to 2 mL Eppendorf tubes on the dry ice and washed with DI water, and stored at −80 °C.

Gene expression analysis

Gene expression analysis was performed according to our earlier reports (Icoglu Aksakal 2019). For the RNA isolation, the RNeasy mini kit (Qiagen, Switzerland) was used with the guidelines of the manufacturer. The qualitative and quantitative analysis of RNA was detected by using Nanodrop ND-100 spectrophotometer. For the case of cDNA synthesis, 100 ng of total RNA was employed with RT^2 First Strand cDNA Synthesis Kit (Qiagen, Switzerland) according to the manufacturer's protocols. Quantitative Real-Time PCR (qRT-PCR) analysis was performed by using SYBR Green (RT^2 SYBR Green master mix) and each PCR analysis was repeated three times. The sequences of primers that were used in the qRT-PCR were given in our earlier reports (Icoglu Aksakal 2019). The actin gene was selected as the housekeeping gene and the $2^{-\Delta\Delta Ct}$ method was employed to determine the change in the gene expression level and the mRNA expression level was represented as fold-change. Finally, the resultant gene expression level and hierarchical analyses were evaluated through Qiagen Data Analysis Center.

Statistical analyses

We employed an independent *t*-test to evaluate the significant differences between control and NP-treated samples in the analysis of the gene expression data. The *p*-value obtained from this analysis is considered to be significant for $^{*}p < 0.05$ and $^{**}p < 0.01$.

Result and discussion

In the first part, we fabricated these NP systems via the proper methods. Figure 1 summarizes the UV-vis absorption spectra, TEM images at different magnifications, and particle size distribution of each NP system. For the case of Che-AgNPs, a sharp absorption maxima was detected at 422 nm indicating the presence of NPs in spherical morphology (Figure 1a). However, in the spectra of Gr-AgNPs, we observed an absorption maxima at a higher wavelength (434 nm) with relatively broader spectra. This issue shows the AgNPs with bigger size and higher size distribution. To further confirm the synthesis of NPs, we performed TEM analysis in detail. Some representative TEM images were given in Figure 2 for Che-AgNPs, and Gr-AgNPs, respectively. In both cases, we observed mostly spherical nanoparticles. We also obtained the size distribution of each NP system by analyzing at least

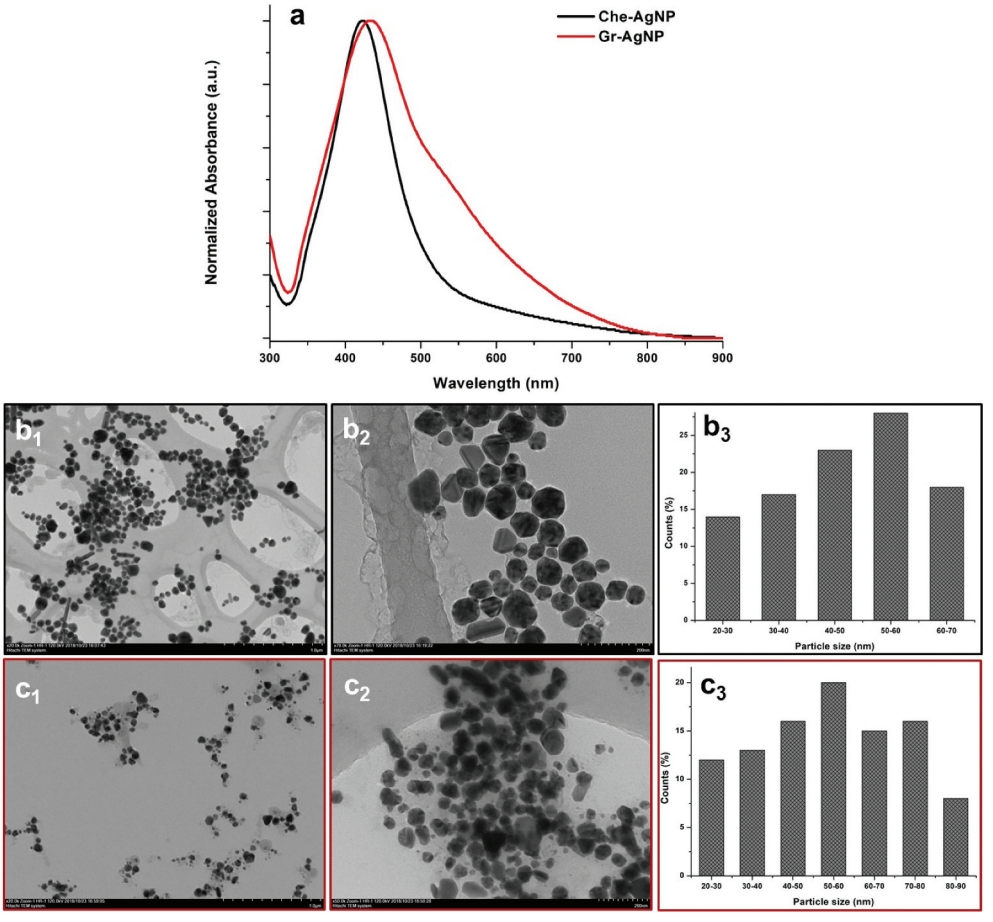

Figure 1. UV-vis absorption spectra of (a), some representative TEM images of Che-AgNPs (b1-2) and Gr-AgNPs (c1-2) and particle size distribution for relevant NP systems (b3 and c3) .

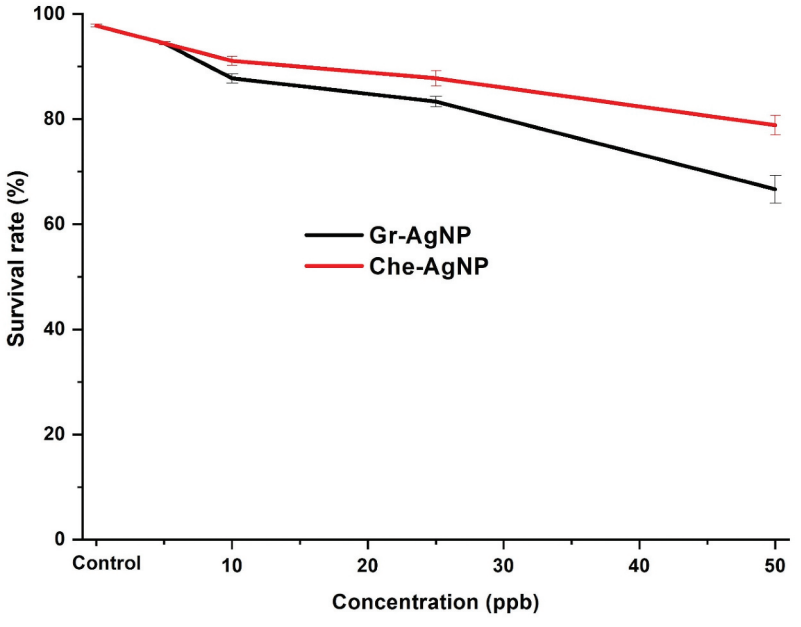

Figure 2. Acute toxicity assay of *D. magna* for each NP system.

100 nanoparticles. The size distribution of Che-AgNPs and Gr-AgNPs was given in Figures b3 and c3, respectively. Che-AgNPs exhibited monomodal size distribution with NP size ranging from 20 to 70 nm and 42 nm of average particle size. However, for the case of Gr-AgNPs, we detected bimodal particle size distribution in the range of 20–90 nm with an average particle size of 53 nm. In the light of the UV-vis and TEM data, we can conclude that both Che-AgNPs and Gr-AgNPs were successfully prepared via proposed synthesis procedures.

After an efficient synthesis of Gr-AgNPs and Che-AgNPs, we employed these NP systems to determine their toxicity in *D. magna*. For this, each NP system was applied to neonates in different concentrations ranging from 5 to 50 ppb. The survival rate of *D. magna* was summarized in Figure 2. From this data, it was detected that both NP systems exhibited dose-dependent toxicity. In comparison to the control group, the exposure of 50 ppb of NPs suspension to *D. magna* led to 19.3% and 31.8% mortality rate for the Che-AgNPs and Gr-AgNPs, respectively. The higher mortality rate of Gr-AgNPs can be attributed to the type of plant components in the surface coverage of NPs due to the presence of plant extract. Phytochemicals carried on the surface of the NPs are likely to play a key role in toxicity (Moore 2006). It was believed that the presence of an organic layer onto the Gr-AgNP system increased its penetration ability to the organism in comparison to Che-AgNPs. The penetration of various NPs with different material, size, shape, and surface coverage material into *D. magna* was shown in many studies (Li et al. 2010; Völker et al. 2013; Sohn et al. 2015; Nasser et al. 2016). It was also determined that each of these parameters has a significant effect on the survival rate of the organism. The effect of the organic layer is required further experimental analysis which will be the subject of our prospective study in the future.

The expression level of some genes related to detoxification and the reproductive system was evaluated in NPs-exposed neonates for 48 h. In comparison to the control groups, exposure to Gr-AgNPs led to a significant increase in the gene expression levels of *cyp360a8* and *hr96* in a dose-dependent manner (Figure 3). It is well-known that the *hr96* gene is homologous to the

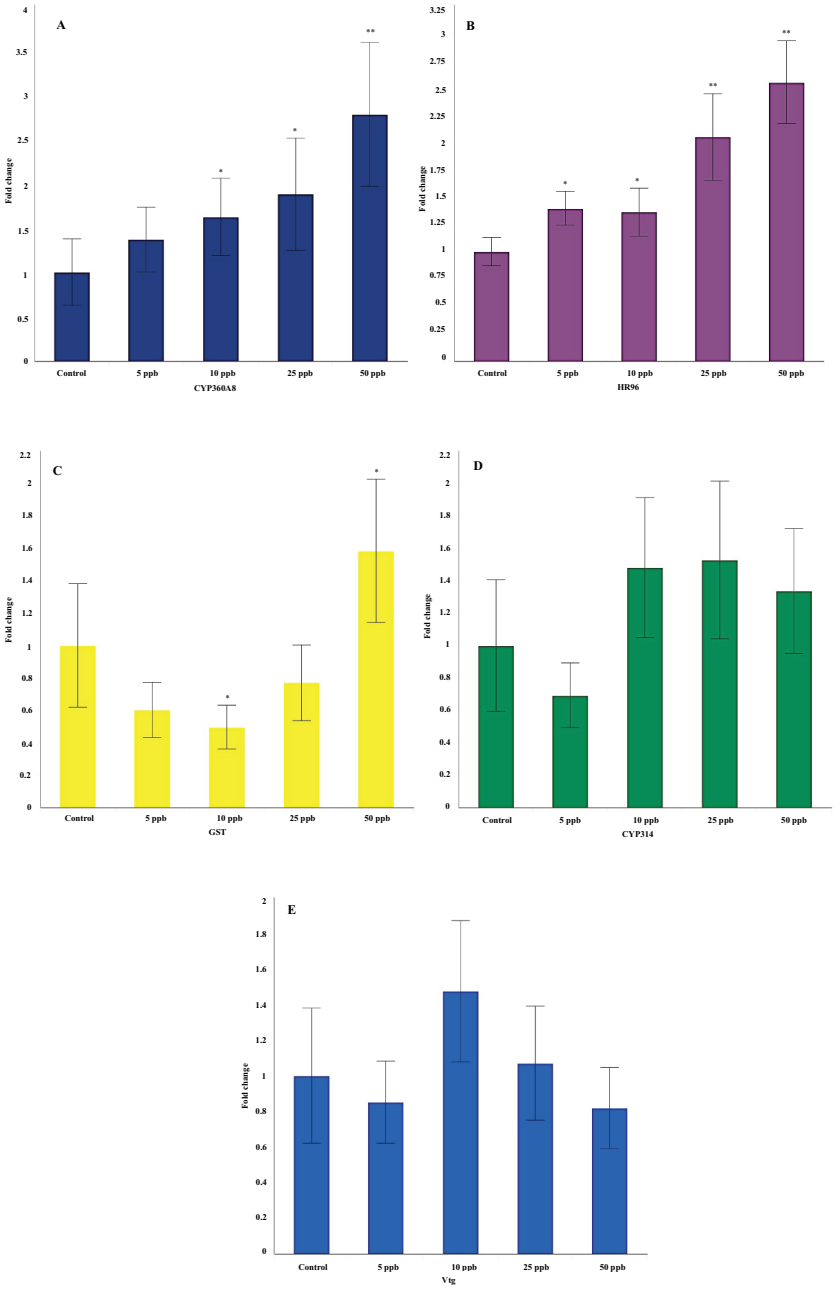

Figure 3. Gene expression (fold change) of *cyp360A8* (A), *hr96* (B), *gst* (C), *cyp314* (D) and *vtg* (E) of *Daphnia magna* after exposure to Gr-AgNP. Data presented as mean±standard deviation (SDM) of three independent replicates. Asterisks indicate significant change with *$p < 0.05$ and **$p < 0.01$.

pxr gene in vertebrates. *pxr* is activated by various xenobiotics in vertebrates and nuclear receptors which play important roles in the regulation of some detoxification enzymes (Kretschmer and Baldwin 2005; Qatanani and Moore 2005; Hernandez et al. 2009). Similarly, *hr96* is a promiscuous receptor and provides important functions in the case of exposure to many toxic substances during the detoxification process. Besides, *cyp360a8* is a homolog gene of *cyp3a*

which is an important member of the P450 superfamily having a significant impact on the detoxification of different substances (Liu et al. 2017a). In our case, the up-regulation of *cyp360a8* and *hr96* genes indicates its effect on catalyzing detoxification of Gr-AgNPs in *D. magna* (Figure 3a,b). *vtg*, a precursor of yolk protein, supplies the nutrient in embryonic development of most invertebrates including many *Daphnia* species. It is also well-known that the production of *vtg* is controlled by estrogen hormones and is an ideal biomarker for the determination of estrogenic compound exposure (Jones et al. 2000; Matozzo et al. 2008). In the present study, there was no change observed in the *vtg* level after Gr-AgNP exposure (Figure 3e). From these data, we can conclude that Gr-AgNP treatment did not create any estrogenic effect in *D. magna* (Hannas et al. 2011).

cyp314 is a member of mitochondrial CYPs and takes part in the conversion of ecdysone to its active form (Rewitz and Gilbert 2008). In arthropods, ecdysteroids hormones act as the activator of ecdysteroids receptor and control the regulation of gene expression relevant to development and reproduction (Liu et al. 2019). Thus, it is highly reasonable to expect that the expression of *cyp314* is well-correlated with reproduction changes. It has been reported that the *cyp314* gene can be used as a biomarker to evaluate the effects of environmental pollutants on *D. magna* molting (Blewett et al. 2017; Liu et al. 2017b, 2019). In this study, no significant change was detected in the expression level of *cyp314* after the Gr-AgNPs application (Figure 3d). It seems that the presence of Gr-AgNPs did not affect the ecdysis in *D. magna*.

Gst is a class of phase II enzymes that provide an important protective effect against reactive oxygen species (Liu et al. 2017a). This enzyme catalyzes the conjugation of glutathione (GSH) with various electrophilic substances and plays a role in preventing oxidative damage by conjugating the break-down products of lipid peroxides to GSH. The expression of *gst* is an informative factor to evaluate the sensitivity of cells against different substances including toxins and nanoparticles in aquatic organisms. In our study, the mRNA level of *gst* was significantly down-regulated in neonates after exposure to 10 ppb Gr-AgNPs (Figure 3c). However, exposure to 50 ppb Gr-AgNPs resulted in a significant up-regulation of this gene. Similar to our results, previous studies reported that AgNPs may induce oxidative stress and modulate the expression of antioxidants including gst as a cellular defense in aquatic organisms (Chae et al. 2009; Park et al. 2015). Our results showed that a lower concentration of Gr-AgNP suppresses *gst* gene expression. This situation may be attributed to the decrease in detoxification processes. The homeostasis of organisms may be temporarily impaired when contaminants newly enter cells, and at low concentrations, the response of defense-related enzymatic activity may not be high enough to trigger gene expression. Depending on the increase in concentration, the expression of genes associated with antioxidant enzymes may increase.

For the case of Che-AgNPs, a significant increase at 25 and 50 ppb concentration was detected in gene expression levels of *cyp360a8* and *hr96* compared to the control groups (Figure 4a-b). However, no remarkable change was observed in *gst, cyp314*, and *vtg* gene expression levels (Figure 4c-e). The heat maps obtained from hierarchical cluster analysis for both the NP system provided gene expression results visually in a different way (Figures 5 and 6).

Conclusion

In this study, we could efficiently synthesize both Che-AgNPs and Gr-AgNPs and used these systems on *D. magna* to determine their aquatic toxicity. The toxicity tests indicated that both NP size and surface coverage materials played crucial roles in the survival rate of the model organism. Also, after exposure to AgNPs, significant changes were observed in the expression of

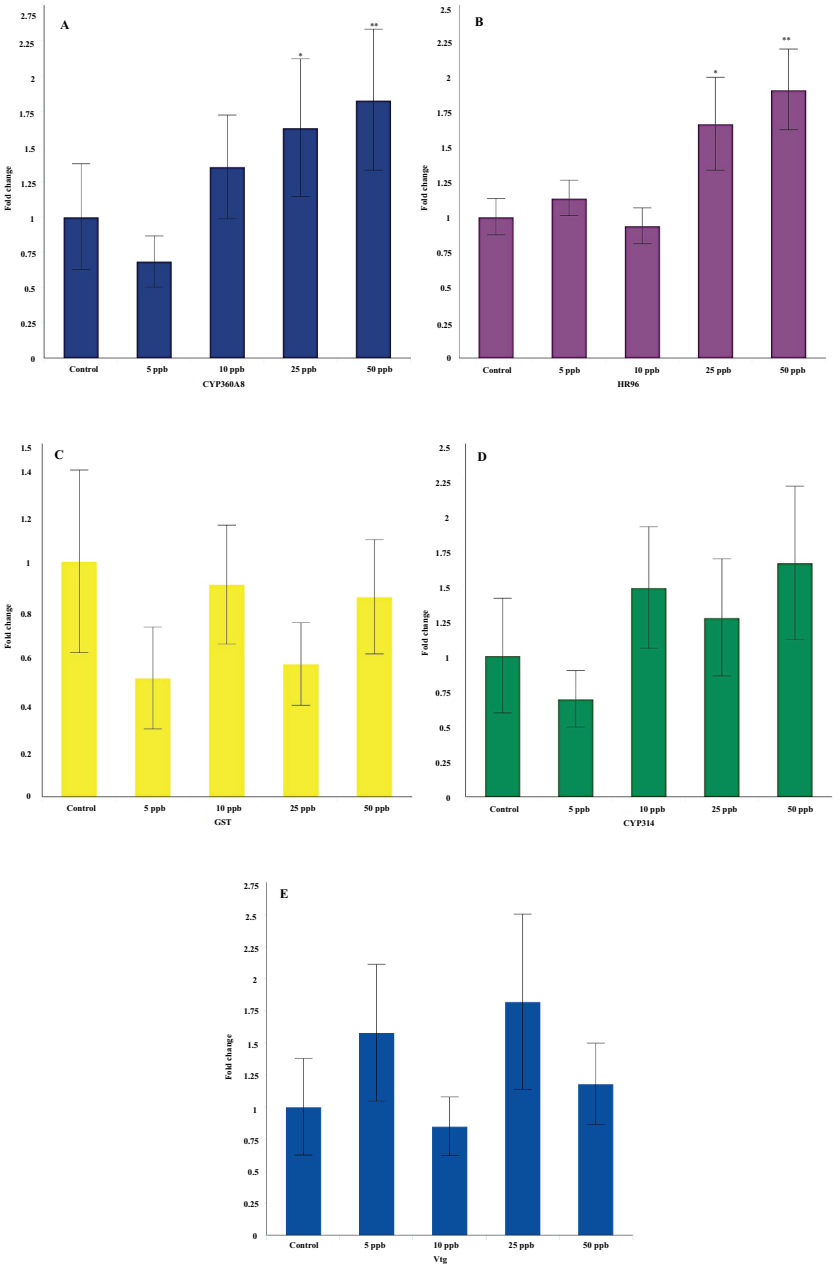

Figure 4. Gene expression (fold change) of *cyp360A8* (A), *hr96* (B), *gst* (C), *cyp314* (D) and *vtg* (E) of *Daphnia magna* after exposure to Che-AgNP. Data presented as mean±standard deviation (SDM) of three independent rep*licates. Asterisks* indicate significant change with $*p < 0.05$ and $**p < 0.01$.

detoxification genes. However, no notable change was detected in the expression of the reproductive system's genes. In the light of these results, we can conclude the synthesis procedure and surface material onto the AgNPs have remarkable effects on the survival rate of *D. magna*.

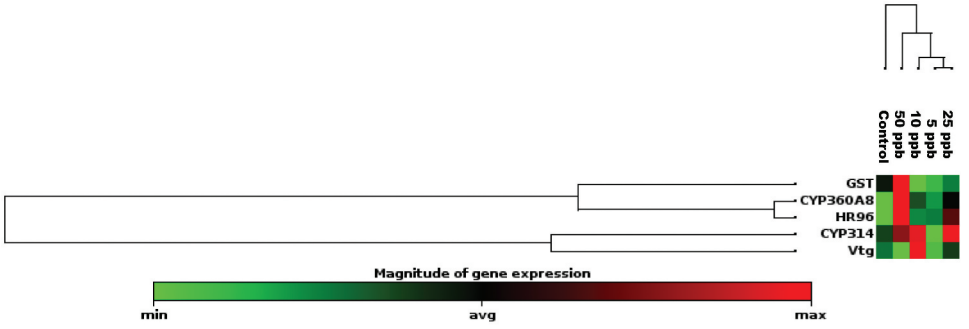

Figure 5. Transcriptional profiles of five selective genes with hierarchical cluster analysis in *D. magna* exposed to Gr-AgNPs for 48 h. A heat map obtained from the GeneGlobe software program demonstrates the mRNA expression levels. Red indicates high expression, while green represents low expression.

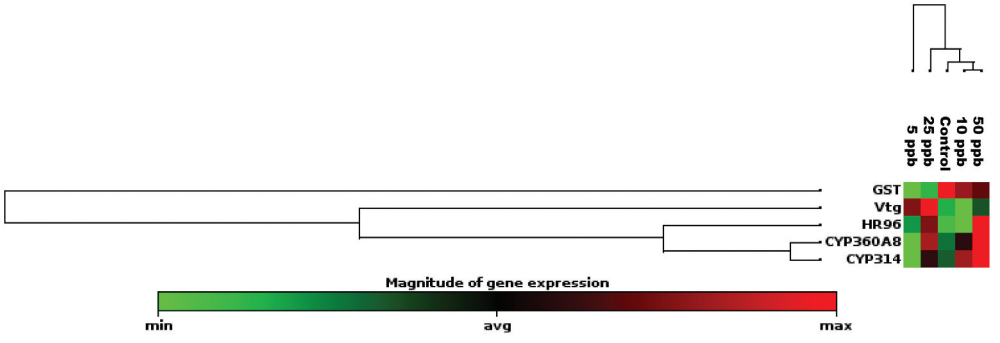

Figure 6. Transcriptional profiles of five selective genes with hierarchical cluster analysis in *D. magna* exposed to Che-AgNPs for 48 h. A heat map obtained from the GeneGlobe software program demonstrates the mRNA expression levels. Red indicates high expression, while green represents low expression.

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Disclosure Statement

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

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