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Rhododendron luteum ve *Rhododendron ponticum* Çiçek Özlerinin *Artemia salina* Larvaları Üzerindeki Potansiyel Toksisitesinin ve Biyokimyasal Aktivitelerinin Karşılaştırmalı Değerlendirilmesi

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ÖZET

Bu çalışmanın odak noktası, *Artemia salina* (tuzlu su karidesi) instar IV larvaları üzerindeki farklı Rhododendron türlerinin (*Rhododendron luteum* ve *Rhododendron ponticum*) farklı konsantrasyonlarda toksisitelerini karşılaştırmalı araştırmaktır. Çünkü, Rhododendron türleri biyoaktif bileşiklerce zengin bir familyaya aittir ki bu da onları oldukça zehirli hale getirmektedir. İki farklı Rhododendron türlerinin instar IV larvaların ölümü oranı üzerinde etkisini, oksidatif stres oluşturma potansiyelini ve larvaların savunma kapasitesini araştırdık. Larvalar hücre kültür plaklarında 48-96 saat boyunca yapay deniz suyunda Rhododendron özütlerine farklı konsantrasyonlarda maruz bırakıldı. Stereomikroskop altında her bir konsantrasyonda ölüm oranı ve morfolojik değişiklikler değerlendirildi. Çalışmamızda, Rhododendron türlerinin *A. salina* instar IV larvaları üzerinde oldukça toksik olduğu ve yine *R. ponticum* türünün *R. luteum*'a göre daha toksik etkili olduğu görülmüştür. Özellikle, 200 µg/mL ve üstü konsantrasyonda her iki Rhododendron türünde hücre hasarını göstren MDA seviyesi anlamılı bir şekilde artmıştır ($P<0.05$). Buna karşın oksidatif stresin etkisini azaltmak için savunma stratejisi olan SOD seviyesi Rhododendron türlerinde artan konsantrasyonla azalmakla birlikte sadece 800-3200 µg/mL konsantrasyon aralığında anlamlıdır ($P<0,05$).

Anahtar Kelimeler: *MDA, oksidatif stres, Rhododendron sp., SOD, toksisite*

Comparative Assessment of Potential Toxicity and Biochemical Properties of *Rhododendron luteum* and *Rhododendron ponticum* Flower Extracts on *Artemia salina*

ABSTRACT

The focus of this study was to comparatively investigate the toxicity of different Rhododendron species (*Rhododendron luteum* and *Rhododendron ponticum*) at different concentrations on instar IV larvae of *Artemia salina* (Brine shrimp). Because *Rhododendron* species belong to a Ericaceae family rich in bioactive compounds, which makes them highly poisonous. We investigated the effect of two different *Rhododendron* species on the mortality rate of instar IV larvae, the potential to induce oxidative stress, and the defense capacity of the larvae. Larvae were exposed to different concentrations of *Rhododendron* extracts in artificial seawater for 48–96 h in cell culture plates. Mortality rate and morphological changes were evaluated at each concentration under a stereomicroscope. In our study, Rhododendron species were found to be highly toxic to *A. salina* instar IV larvae and *R. ponticum* was more toxic than *R. luteum*. Particularly, the level of MDA, which indicates cell damage, increased significantly in both Rhododendron species at a concentration of 200 µg/mL and above ($P<0.05$). On the other hand, the level of SOD, which is a defense strategy to reduce the effect of oxidative stress, decreased with increasing concentration in Rhododendron species, but was significant only in the concentration range of 800-3200 µg/mL ($P<0.05$).

Keywords: *MDA, oxidative stress, Rhododendron sp. SOD, toxicity*

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1. GİRİŞ

Rhododendron species are the biggest genus of the Ericaceae family and are perennial plants with a wide distribution (Clinton &Vose, 1996; Neary et al., 1980; Çolak, 1997). *Rhododendron* species have a wide distribution on the northern coasts of Turkey. The two most common species in Turkey are *Rhododendron luteum* and *Rhododendron ponticum*. *R. luteum* contains the poisonous substance grayanotoxin in its leaves and flowers. It is also used in traditional medicine as a pain reliever for rheumatic pain, a diuretic, and fungal foot infections, and it also has strong antioxidant and anticancer effects (Popescu & Kopp, 2013). *R. ponticum* is a species with allelopathic effects and contains ericolin and grayanotoxin glycosides in its flowers. It significantly affects the elements of the soil in the region where it is located and reduces the pH value of the soil. In addition, it has two types of invasion strategies: slow invasion by vegetative means and rapid invasion by generative means (Kulaç, 2004). In general, the main components of Rhododendron species are hydrocarbons, esters, alcohols and ketones (Tasdemir et al., 2023). Studies to date have shown the antimicrobial, antioxidant, anti-inflammatory, analgesic, antidiabetic, hepatoprotective, immunomodulatory and antineoplastic effects of Rhododendron species (Demir & Aliyazicioglu 2016; Popescu and Kopp, 2013). For example, one study evaluated the cytotoxic effects of *R. ponticum* L. extract on prostate carcinoma and adenocarcinoma cell lines (Bilir et al., 2018a). Also, the cytotoxic and anti-proliferative effects of *R. ponticum* L. extract on rat glioma cell line were investigated (Bilir et al., 2018b).

In the present study, the toxic effect of *Rhododendron* species was evaluated on the aquatic zooplankton *Artemia salina*. *A. salina* is the indispensable test organism for aquatic ecotoxicity tests. It has been used in aquatic ecotoxicity studies so far and will continue to be used in the future (Nunes et al., 2006; Dağlıoğlu & Çelebi, 2015; Dağlıoğlu et al., 2016a,b; Dağlıoğlu et al., 2023). The *A.salina* lethality analysis was chosen in this study because it is a very suitable test system for monitoring the biological activities of various plant species. This method is very useful for the preliminary assessment of toxicity of plant extracts. Some of its most important advantages are that it is a fast and simple method and that laboratory requirements are relatively low. However, there are some important points to consider, namely that optimized experimental conditions such as pH of the environment, temperature, ventilation, salinity and light must be at optimum levels.

The aim of this study was to assign the toxic effects of *R. luteum* and *R. ponticum* extracts gathered from Ordu province on the aquatic indicator organism *A.salina* in a comparative manner.

2. MATERIAL AND METHODS

2.1. Preparation of the Plant Extract

Rhododendron luteum (yellow rhododendron) and *Rhododendron ponticum (purple rhododendron)* flowers were collected from Turnalık plateau in Ordu province and dried in an oven at 25 °C for one week and then turned into powder.Next, it was mixed with 6 g of plant powder and 60 mL of ethanol and then vortexed. It was incubated at 50 ^{o}C and 150 rpm on a shaker for 36 h. After incubation, these samples were centrifuged at 4000 rpm for 10 minutes and the supernatant was drained with Whatman filter paper (Whatman, No. 3, Sigma-Aldrich) and the ethanol was removed in the evaporator. The obtained plant extracts were dissolved by adding 6 mL dH_2O. The acquired product was stored at -20°C for use in experiments.

2.2. Acute Toxicity Test

For the experiments, *A. salina* cysts, which are primary consumer zooplankton species living in artificial seawater environment, were supplied by a commercial companyLarvae were hatched by adding 4 g of *A. salina* cysts to 1 L of filtered and sterilized artificial natural seawater and incubating at 28˚C, pH 7.6, under continuous aeration for 24 h. The toxicity of Rhododendron extracts on *A.salina* larvae was determined in the concentration range of 0, 25, 50, 100, 200, 400, 800, 1600 ve 3200 µg/mL. Experiments were performed in 24-well cell culture dishes with 10 individuals per well in 3 replicates. Continuous ventilation was provided during the experiment. The number of dead and living organisms was enumerated under a binocular microscope at 48 and 96 hour time periods, and the arithmetic average of the values was taken. As a result of the 48 and 96 hour experiment, the number of individuals dying and the percentage of deaths for each concentration were determined.

2.3. Biochemical Analyses

For each biochemical analysis, approximately 1800 *A. salina* larvae were exposed to *R. luteum* and *R. ponticum* extracts at the specified concentrations.

2.4. Super Oxide Dismutase (SOD) Activity

SOD activity was performed with reference to the protocol represented by Ateş et al., (2013). The collected larvae were homogenized in 1 mL of 0.5 M potassium phosphate buffer (pH 7.5) using a glass homogenizer (ILDAM, Turkey). Homogenized specimens were centrifuged at 15,000 rpm for 15 minutes and the supernatant was gathered. Then, 50 mM (1.3 mL) Na_2CO_3 buffer (pH 10), 100 µL Triton X-100, 20 mM (100 µL) hydroxylamine hydrochloride and 96 mM (500 µL) NBT were joined to 70 µL of the supernatant to initiate the reaction. These solution was incubated for 20 min at 37 °C in the presence of light. After incubation, it was measured at 540 nm in UV-VIS spectroscopy. The decrease in absorbance at 540 nm is a measure of the reduction of nitro blue tetrazolium chloride (NBT) by the SOD enzyme (Sugantharaj David et al., 2017).

2.5. Malondialdehyde (MDA) Activity

MDA activity, which is one of the lipid peroxidation products, is based on the reaction of thiobarbituric acid (TBA) and MDA to form a red colored compound. The obtained red-pinkish pigment was measured in a spectrophotometer at 532-535 nm. Tetramethoxypropane was used as standard. MDA activity was performed according to the protocol described by Ates et al., (2013a). The collected larvae were homogenized in 2 mL of 0.5 M potassium phosphate buffer (pH 7.2) with a glass homogenizer (ILDAM, Turkey). Homogenized samples were centrifuged at 6000 rpm for 10 minutes and the supernatant was collected. Then, 10 µL of BHT, 0.25 mL of phosphoric acid reagent and 0.25 mL of TBA reagent were added to 0.25 mL of the supernatant in a sterile bottle. The obtained reaction mixture was incubated at 90°C for 1 hour and centrifuged after cooling at 13,000 rpm for 10 minutes to sediment the suspended tissue. These mixture was measured at 532 nm in UV-VIS spectroscopy.

2.6. Statistical Analyzes

The obtained data were recorded as standard deviation ± mean. SPSS version 26 was applied to comparatively evaluate the effects of *Rhododendron sp.* flower extracts on the toxicity of *A. salina.* Data were examined according to normal distribution, and data that did not conform to normal distribution were normalized using the Kolmogorov-Smirnov test. One-way ANOVA was performed using Tukey's

multiple range test to compare significant mean differences between the control and treatment groups. Significant differences in mortality rate, MDA and SOD tests were accepted at P<0.05.

3. RESULTS

3.1. Biochemical Results

In this study, test organism *Artemia salina* instar IV (4th stage) larvae were used to reveal the toxic effects of *Rhododendron luteum* (Rl) and *Rhododendron ponticum* (Rp) flower extract (Fe). Fig. 1 indicates concentration-dependent increases in mortality of instar IV larvae following exposure to RlFe and RpFe solutions. No deaths were observed in the control groups throughout the experiment. Mortality rates following exposure to RlFe and RpFe solutions varied greatly depending on exposure duration and exposure concentration. Overall, *R. ponticum* exhibited more toxic effects at both 48 and 96 h exposure compared to *R. luteum*. When looked at individually, high mortality was observed even at low concentrations of RpFe. At 96 h of exposure, 100% mortality occurred at the highest RpFe concentration (3200 µg/mL) and 30% mortality occurred at the lowest concentration (25µg/mL) (Fig. 1). No mortality was observed at 25µg/mL over 48 hours of exposure. The mortality rate of larvae exposed to different RpFe concentrations for 48 and 96 h increased significantly with increasing concentration (P<0.05). *In R. luteum*, no death was observed in the concentration range of 0-200 µg/mL after 48 h of exposure. The death rate was 70% at 3200 µg/mL . Increasing the RlFe concentration from 400 to 3200 µg/mL increased the mortality rate of larvae, but this rate was not significant at higher concentrations (P>0.05). There was a significant difference in mortality among concentrations at 96 hours of exposure (P<0.05). This difference was present among all concentration groups.

When we evaluate all these data, it is seen that the extracts of Rhododendron species prepared at the same concentration have quite different toxic effects from each other, but *R. ponticum* species is more toxic than *R. luteum*.

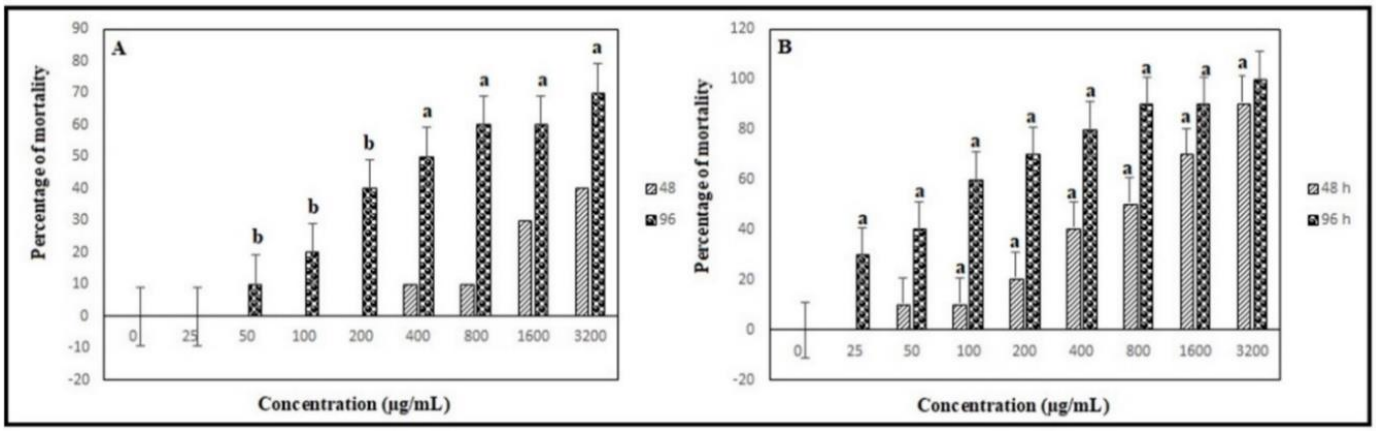

Figure 1. Mortality rates of *R. luteum* and *R. ponticum* flower extract depending on exposure times A) *R. luteum* and B) *R. ponticum.* The values are expressed as mean ± standard deviation (SD). Values significantly different from the control group are shown in lower case letters. (ANOVA, P<0.05).

Changes in the morphology of instar IV larvae exposed to *R. luteum* and *R. ponticum* flower extract were observed. In Fig. 2A, we see that the control group *A. salina* larvae have a very healthy morphology. However, the morphology of larvae exposed to RlFe and RpFe solutions changed. In Fig. 2B, the extremities of larvae exposed to RlFe for 48 h were damaged. As the red arrow indicates, the intestine is

full, indicating that it is feeding on RlFe. Also, the green arrow shows the damaged antenna of the larva. In Fig. 2C, the morphology of larvae exposed to RpFe for 48 h is quite distorted. Even though the intestine is fragmented, it is seen to be full.

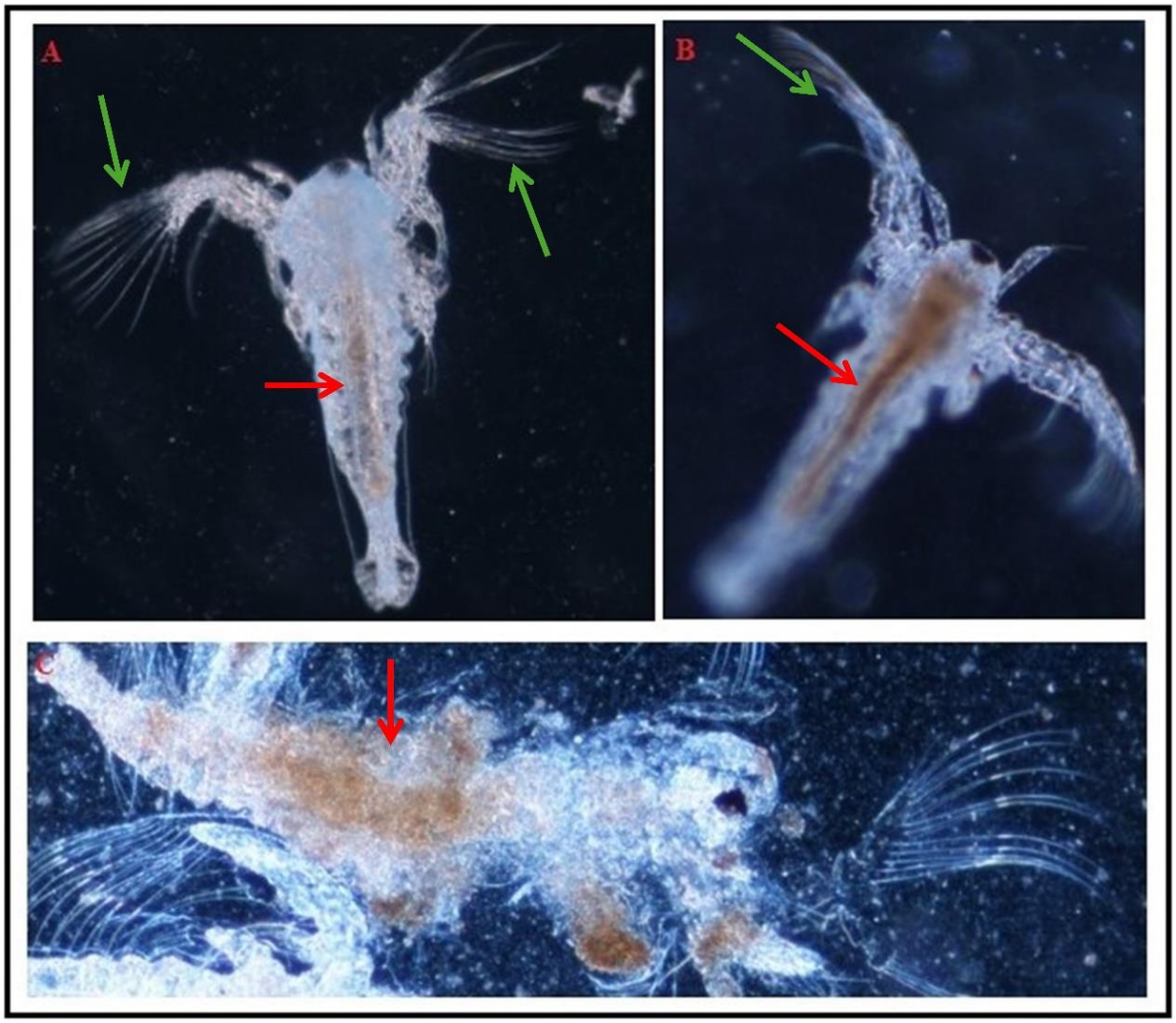

Figure 2. Phase-contrast microscopy image of larvae exposed to
A) control B) *Rhododendron luteum* and C) *Rhododendron ponticum* flower extract.
Red arrow indicates the intestine, green arrow indicates the antennae.

Phase-contrast microscopy image of larvae exposed to control Rhododendron luteum and Rhododendron ponticum flower extract. In this study, MDA, which we investigated as a biomarker, is a metabolite produced from lipid peroxidation and was used as an marker of oxidative damage in the cell membrane. The SOD antioxidant enzyme is an antioxidant that catalyzes the decomposition of reactive oxygen species (ROS) and protects organisms from the negative effects of oxidative stress (Cazenave et al., 2006). In our study, in general, when *A. salina* instar IV was treated with RlFe and RpFe, MDA activity increased and SOD activity decreased with increasing extract concentrations. When examined separately, after 48 h of exposure to RlFe, MDA production in the concentration range of 0-200 µg/mL was not produced at a significant grade compared to the control group ($P>0.05$). MDA content increased significantly at concentrations of 400 µg/mL and above, and a significant difference was sighted between

the concentration groups (P<0.05). At 96 h of exposure, MDA production at 0-100 µg/mL concentration was not at a significant difference compared to the control group (P>0.05). Above 200 µg/mL, MDA content increased significantly and a significant difference was observed between the concentration groups (P<0.05). SOD activity generally decreased with increasing concentration, and a significant difference was observed compared to the control group at 25, 1600, 3200 µg/mL at 48 h (P<0.05). In 96 h application, a significant difference was observed only at 1600 and 3200 µg/mL (P<0.05). After 48 and 96 h of RlFe exposure, MDA content increased significantly and significant differences were observed among the concentration groups (P<0.05). After 48 and 96 h of RpFe exposure, MDA content increased significantly with increasing RpFe concentration (P<0.05). However, MDA production in the range of 50-100 µg/mL was not significant at both exposure times (P>0.05). SOD activity decreased with increasing concentration, but this decrease was significant only in the concentration range of 800-3200 µg/mL (P<0.05).

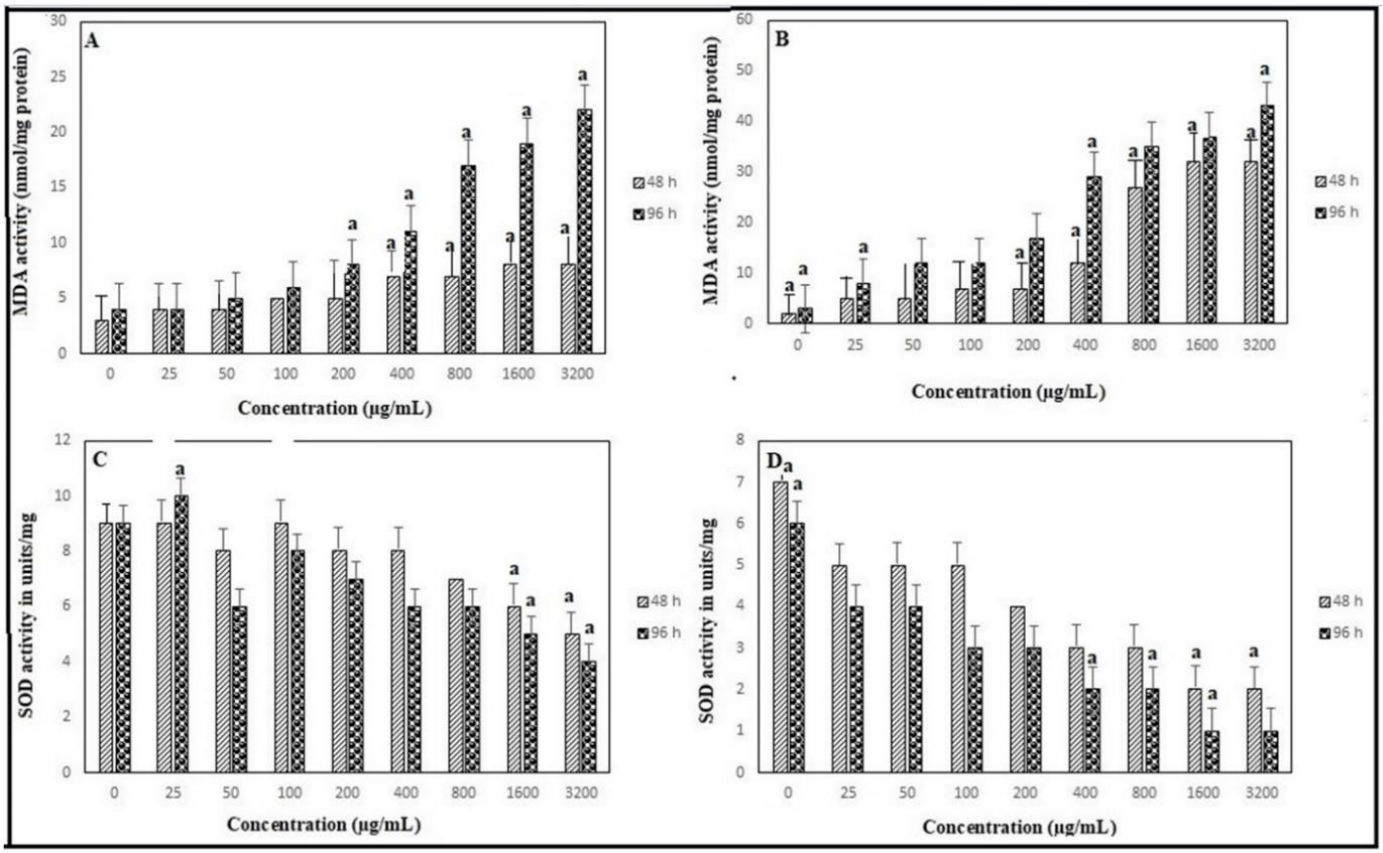

Figure 3. SOD and MDA contents determined in *A. salina* instar IV a exposure to RlFe and RpFe for 48 and 96 h.

A) MDA activity of *R. luteum,* B) SOD activity of *R. luteum* C) MDA activity of *R. ponticum* D) SOD activity of *R. ponticum.* The values are expressed as mean ± standard deviation (SD). Values significantly different from the control group are shown in lower case letters. (ANOVA, P<0.05).

4. DISCUSSION

The selection of indicator organisms for toxicity tests is a very important factor. In these tests, the morphological changes observed in adults and larvae, their movement abilities and most importantly, mortality rates are the most indispensable evaluation criteria. The use of *A. salina* as an indicator in ecotoxicology, the practicality of laboratory culture and the easy obtainment of cysts make toxicity tests highly successful and reproducible. The aim of the present study was to compare the toxicity of Rhododendron species (*R. luteum* and *R. ponticum*), known to be poisonous, on the aquatic indicator organism *A. salina*. Rhododendron species have been characterized for their toxic components in previous studies (Gunduz et al., 2008; Jansen et al., 2012). So far, 208 compounds, mostly flavonoids and terpenoids, have been isolated from Rhododendron species. Some of these compounds are quercetin, hyperoside, farrerol and polistachoside (Hu & Xiao, 1989). Therefore, Rhododendron species create different toxicities with their rich contents. In this study, the toxicity of two different Rhododendron species growing in the plateaus of Ordu (Türkiye) was investigated and compared with each other.

Mortality rates of *A. salina* instar IV larvae were observed to be considerably higher in *R. ponticum* compared to *R. luteum.* This increase occurred depending on concentration and time. Previous studies have also noted that mortality increases depending on the concentration of the substance exposed and the time. Wang et al. (2017) observed that the increase in mortality rate increased in a concentration-dependent manner when they exposed instar I, II, and III larvae of A. salina to α-Fe_2O_3 nanoparticles. In another study, concentrations causing 50% mortality were detected in *A. salina* larvae exposed to mycotoxins such as aflatoxin, diacetoxysirpenol, gliotoxin, ochratoxin, and sterigmatocystin for 16 h (Harwig & Scott, 1971). The toxicity of Iranian medicinal plants (*Plantago major, Artemisia maritima, Mentha piperata* ve *Borrago officianalis*) was tested *A. salina* and *A. uramiana* lethality tests. All plant extracts except *B. officinalis* caused 100.0% mortality in both Artemia at 1000 µg/ml (Mirzaei & Mirzaei, 2013).

Plant bioactive compounds such as phenolics and flavonoids, which are abundant in Rhododendron species, can cause toxicity to cells (George & Abrahamse, 2019). Some studies have reported that toxic effects on *A. salina* are due to oxidative stress (Ates et al., 2013a; 2013b). The main indicators of oxidative stress are the changing levels of some metabolites, namely MDA and ROS, and antioxidant enzyme amounts in the cells (Mesarič et al., 2015; Ates et al., 2015; Zhu et al., 2016). MDA is a metabolite derived from lipid peroxidation and has been on a large scale used as an indicator of oxidative damage and oxidative stress in membranes (Ates et al., 2013a; 2013b; 2015). In the present study, both *R. luteum* and *R. ponticum* flower extracts caused cell damage in *A. salina* instar IV larvae. RpFe caused much more cell damage than RlFe when evaluated at the same concentrations. However, this damage increased significantly at 96 h of exposure compared to 48 h. Furthermore, cell damage increased at 48 and 96 h with increasing concentration in both extracts. Results parallel to our study have been recorded in other studies. For example, MDA content in *A. salina* instar III larvae exposed to α-Fe2O3-NP suspensions increased significantly depending on the α-Fe2O3-NP dose, suggesting that the toxic effects were through by oxidative stress (Wang et al., 2017). MDA biomarkers were used as sign of oxidative stress in *A. salina* exposed to zinc oxide nanoparticles (ZnO NPs) and the amount of MDA increased with increasing zinc concentration (Ateş et al., 2020). The toxicity of polystyrene nanoplastics (PSNPs) in *A. salina* exposed to nanopermethrin (NPER) was demonstrated by the MDA content and at high concentration, a very high MDA level was reported (Kamalakannan et al., 2024). In this study, determination of SOD activity is of great importance for the evaluation of the antioxidant capacity of the

test organism. The decrease in the activity of antioxidant enzymes is the first sign of oxidative stress (Varó et al., 2019). In our study, induction of oxidative stress by RpFe and RlFe was detected by a reduce in the amount of SOD. The amount of SOD decreased significantly with increasing concentration, especially in *R. ponticum* flower extract. At high concentrations, this reduce was significant compared to the control group ($P<0.05$). It also decreased with increasing concentration in *R. luteum*. However, this reduce is less than that of *R. ponticum* flower extract. Similar results were observed in toxicity studies with *A. salina*. For example, in *A. salina* exposed to PSNPs, SOD activity decreased with increasing concentration (Kamalakannan et al., 2024). For *A. salina* Instar I and II larvae exposed to Fe_3O_4 nanoparticles, a gradual increase in SOD activity was noted followed by a decrease. Researchers reported that SOD activity can be inhibited by high ROS levels (Zhu et al., 2017).

5. CONCLUSION

In this study, brine shrimp toxicity results showed that Rhododendron species showed quite different toxic effects on *A. salina* at the same concentration and 48 and 96 h treatment. However, *Rhododendron ponticum* species has a much higher toxic effect than *Rhododendron luteum*. This situation was clearly seen in both mortality rates and biochemical analyses. SOD activity decreased in cells that could not cope with the increase in MDA, which caused oxidative stress. This toxic effect may be caused by plant bioactive compounds such as phenolics and flavonoids, which are abundant in Rhododendron species. Additionally, *A. salina* is a very suitable test organism for evaluating the toxicity of plant extracts.

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