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In VIVO MODULATORY EFFECT of MALACHITE GREEN on CYTOCHROME P450 AND ANTIOXIDANT ENZYME ACTIVITIES in RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) LIVER

MALAHİT YEŞİLİNİN GÖKKUŞAĞI ALABALIĞI (*ONCORHYNCHUS MYKISS*) KARACİĞERİNDE SİTOKROM P450 VE ANTİOKSİDAN ENZİM AKTİVİTELERİ ÜZERİNE İN VİVO MODÜLATÖR ETKİSİ

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ABSTRACT

Malachite green is a mutagenic and carcinogenic chemical used as a dye in textile and paper industry and in fish farms against ectoparasites, bacterial and fungal infections. Aquatic organisms are at risk of malachite green exposure. In this study, our aim was to determine the effect of two different doses of malachite green on cytochrome P450 and antioxidant system in rainbow trout (*Oncorhynchus mykiss*). For this purpose, 24 fish were randomly divided into three groups: the control, the 0.1 mg/L malachite green administration group and the 0.5 mg/L malachite green administration group. 7-ethoxyresorufin-O-deethylase activity (EROD) in the 0.5 mg/L malachite green administration group was significantly higher than that in the control group. 7-pentoxyresorufin O-dependentylase (PROD) activities increased with increasing doses of malachite green. Catalase (CAT) and glutathione reductase (GR) activities in the 0.1 mg/L malachite green administration group were significantly higher than those in the control group. No statistically significant differences were found in erythromycin N-demethylase (ERND) and glutathione S-transferase (GST) activities among the groups. The results of this study clearly demonstrate that malachite green has a modulatory effect on EROD, PROD, CAT, and GR activities in rainbow trout.

Keywords: Antioxidant enzyme activities, Cytochrome P450, Malachite green, Rainbow trout (*Oncorhynchus mykiss*), Xenobiotic metabolizing enzymes.

ÖZ

Malahit yeşili, tekstil ve kâğıt endüstrisinde boya olarak ve balık çiftliklerinde ektoparazitlere, bakteriyel ve fungal enfeksiyonlara karşı kullanılan mutajenik ve karsinojenik bir kimyasaldır. Sucul organizmalar malahit yeşiline maruz kalma riski altındadır. Bu çalışmada amacımız gökkuşağı alabalığında (*Oncorhynchus mykiss*) iki farklı konsantrasyonda malahit yeşilinin sitokrom P450 ve antioksidan sistem üzerine etkisini belirlemektir. Bu amaçla, 24 balık üç gruba rastgele ayrılmıştır: kontrol, 0,1 mg/L malahit yeşili ile muamele edilen grup ve 0,5 mg/L malahit yeşili ile muamele edilen grup. 0,5 mg/L malahit yeşili ile muamele edilen grupta 7-etoksiresorufin O-deetilaz (EROD) aktivitesi, kontrol grubundan anlamlı düzeyde daha yüksektir. 7-pentoksiresorufin O-dependentilaz (PROD) aktivitesi artan malahit yeşili dozu ile artmıştır. 0,1 mg/L malahit yeşili ile muamele edilen grupta katalaz (CAT) ve glutatyon redüktaz (GR) aktiviteleri, kontrol grubundan anlamlı düzeyde daha yüksektir. Eritromisin N-demetilaz (ERND) ve glutatyon S-transferaz (GST) aktivitelerinde gruplar arasında istatistiksel olarak anlamlı fark bulunmamıştır. Bu çalışmanın sonuçları malahit yeşilinin gökkuşağı alabalığında EROD, PROD, CAT ve GR aktiviteleri üzerinde modülatör bir etkiye sahip olduğunu açıkça göstermektedir.

Anahtar Kelimeler: Antioksidan enzim aktiviteleri, Sitokrom P450, Malahit yeşili, Gökkuşağı alabalığı (*Oncorhynchus mykiss*), Ksenobiyotik metabolize eden enzimler

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Introduction

Malachite green (4-{[4-(Dimethylamino) phenyl] (phenyl)methylene}-N,N-dimethyl-2,5-cyclo hexadien-1-iminium) is an organic chemical, readily soluble in water, and used to color wool, silk and cotton in textile industry. It is also used in aquaculture as a therapeutic chemical due to its anti-parasitic and anti-fungal effects. It was previously used to give color to foods. However, its toxic and carcinogenic effects have been identified, leading to its prohibition in edible by the FDA in the US and EU countries (Dutta et al., 2024; Zu-yi et al., 2024). Nevertheless, recent studies have shown that fish are still exposed to malachite green (Hakami et al., 2021; Gharavi-nakhjavani et al., 2023; Lemos et al., 2023; Fakhri et al., 2025).

Malachite green is rapidly absorbed and metabolized to leucomalachite (Lemos et al., 2023). A study on the accumulation of malachite green in different organs revealed that the highest amount of malachite green is found in the liver (Gharavi-nakhjavani et al., 2023; Srivastav et al., 2016; Sinha et al., 2021). The toxic effect of malachite green has been shown in the liver tissue of rats, mice, and several fish species (Culp et al., 1999; Das et al., 2013; Hassan et al., 2014; Sinha et al., 2021). Oxidative biotransformation products, such as malachite green N-oxide and malachite green N-demethylated derivatives, have been detected in edible fish tissues (Doerge et al., 1998).

In vivo studies the biotransformation reactions of malachite green are limited, and contradictory results regarding its effects have been reported in the literature. Nebbia et al. (2017) reported the inhibitory effect of malachite green on GST and CYP1A enzyme activities with *in vitro* studies. In their study, they highlighted the necessity of *in vivo* studies to confirm their in *vitro* results. Phase I and Phase II enzymes play roles in the biotransformation reactions of xenobiotics. Most organic pollutants, including polycyclic aromatic hydrocarbons, solvents, pesticides, drugs, cosmetics, and food preservatives, are initially metabolized by phase I reactions. Most of the phase I reactions are catalyzed by cytochrome P450-dependent mixed function oxidases (Esteves et al., 2021). Cytochrome P450s (CYPs) have roles in detoxification reactions or the conversion of non-toxic chemicals to toxic forms (Veith & Moorthy, 2018; Esteves et al., 2021). CYP1A, one of the cytochrome P450 subfamilies metabolizes the initial metabolic reaction of many organic chemicals, including polycyclic aromatic hydrocarbons and polychlorinated biphenyls. It has also been demonstrated in many different organisms that the level of cytochrome P4501A increases by these organic substances (Goksoyr, 1995; Bozcaarmutlu et al., 2015; Rabuffetti et al., 2024; Sadauskas-Henrique et al., 2024). CYP3A and CYP2B are generally involved in detoxification reactions. CYP3A is responsible for the metabolism of the majority of drug molecules in the body (Xu et al., 2005). CYP2B also plays a role in the metabolism of endogenous substances such as androstenedione and testosterone (Nemoto & Sakurai, 1995).

Xenobiotics are generally further metabolized by phase II enzymes. These enzymes catalyze conjugation reactions of chemicals. Conjugation reactions convert molecules from their lipid-soluble forms to water-soluble forms which are then excreted from the body (Tew & Ronia, 1999; Rabuffetti et al., 2024). One of the phase II enzyme families is glutathione S-transferase (GST), which is involved in conjugation reaction between reduced glutathione and xenobiotics (Tew & Ronia, 1999). Additionally, free radicals are formed during metabolic and biotransformation reactions of xenobiotics (Tew & Ronia, 1999). Free radicals react with macromolecules in the cell, leading to protein or DNA damage. The effects of free radicals are eliminated by an antioxidant system (Vilchis-Landeros et al., 2024). Proteins in the antioxidant system include catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase enzymes.

In this study, our aim was to determine the *in vivo* effect of two different doses of malachite green on xenobiotic metabolizing enzyme activities, including CYP1A-associated 7-ethoxyresorufin-O-deethylase (EROD), CYP3A-associated erythromycin N-demethylase (ERND), CYP2B-associated 7-pentoxyresorufin O-depentylase (PROD), and glutathione S-transferase (GST) in the liver of rainbow trout (*Oncorhynchus mykiss*). In addition, the *in vivo* effect of malachite green on antioxidant system was determined by measuring catalase (CAT) and glutathione reductase (GR) activities in the liver of rainbow trout (*Oncorhynchus mykiss*). The involvement of CYP1A on the metabolism of malachite green was also supported through *in vitro* studies.

Methods

Fish Material and Treatment

The ethical approval for the animal-based studies was obtained from the Animal Experiments Local Ethics Committee of Bolu Abant Izzet Baysal Unıversıty University (Process number: 2018/21). Rainbow trout samples were purchased from a local trout farm in BoluTürkiye. The fish

were kept in a separate flow-through pond for 3 days before sampling. A total of 24 fish (26 ± 0.2 cm) were sampled from this pond and transferred to Fish Biology Laboratory in the Bolu Abant Izzet Baysal University. They were then randomly separated into three groups in 200 L aerated fiberglass tanks. The groups were named as control, 0.1 mg/L malachite green treatment group, and 0.5 mg/L malachite green treatment group. Malachite green oxalate was purchased from Merck KGaA (Darmstadt, Germany). The temperature of tanks was maintained constant at 11-12 °C. The tanks were drained and refilled every twelve hours. The fish were kept in 12 hours dark and 12 hours light cycle. Liver samples of fish treated with 0.5 mg/L malachite green were taken at the end of 24 hours by cervical dislocation and placed in liquid nitrogen for freezing. Liver sample of fish treated with 0.1 mg/L malachite green, and the control groups were taken at the end of 54 hours by cervical dislocation and placed in liquid nitrogen for freezing. They were then stored in a deep freezer at -80 °C until used.

Preparation of Microsomes and Cytosols

The liver tissue fractions of rainbow trout liver were prepared using the method described by Arınç & Şen (1993) with some modifications. Each tissue was prepared individually. The tissue pieces were homogenized with homogenization solution (1.15% KCl solution containing 1.0 mM EDTA pH 7.4, 0.1 mM PMSF and 0.25 mM ε-ACA) at a volume equal to 4 times the weight of the tissues. The homogenate was centrifuged at 10000×g for 20 minutes at 4°C. Microsomes and cytosols were then prepared as reported by Bozcaarmutlu et al. (2015). Finally, microsomes were resuspended in 25% glycerol containing 1.0 mM EDTA, pH 7.4, at a volume of 1.0 mL for each gram of rainbow trout liver tissues. Microsomes and cytosols were stored in a -80 °C deep freezer. Protein concentrations of microsomes and cytosols were determined using the Lowry Method (Lowry, 1951).

Enzyme Activity Measurements

The details of the procedures used for enzyme activity measurements were explained in previous reports (Bozcaarmutlu & Arinç, 2008; Bozcaarmutlu et al., 2020). Cytochrome P4501A associated 7-ethoxyresorufin-O-deethylase (EROD) and cytochrome P4502B associated 7-pethoxyresorufin-O-depenthylase (PROD) activities of fish liver microsomes were measured as described by Burke & Mayer (1974) and Arinc & Şen (1993). The optimum conditions for maximum activities were determined for rainbow trout in our laboratory through characterization studies. NADPH generating system was used in these activity measurements and prepared as reported by Bozcaarmutlu et al. (2015). A typical reaction medium contained 6.5 µM 7-ethoxyresorufin for EROD and 6.5 µM 7-penthoxyresorufin for PROD. The reaction was initiated with the addition of substrate of each reaction and followed at 25°C for 5 minutes for EROD and 10 minutes for PROD in a spectrofluorometer at 535 nm (excitation) and 585 nm (emission) wavelengths. Both EROD and PROD activities were determined by using standard calibration curve of resorufin.

Cytochrome P4503A associated erythromycin N-demethylase (ERND) activities in fish liver microsomes were measured as described by Cochin & Axelrod (1959) with some modifications. A typical assay mixture for rainbow trout contained 1 mM erythromycin. The reaction was carried out at 25°C for 15 minutes with shaking in a water bath. The amount of formaldehyde produced at the end of enzyme catalyzed reaction was measured by the method of Nash (1953). Standard calibration curve of formaldehyde was used to calculate the specific ERND activities.

Total glutathione S-transferase (GST) activities in rainbow trout liver cytosols were measured as described by Habig et al. (1974) with some modifications. A typical reaction mixture contained 3.0 mM GSH and 1.0 mM CDNB. The reaction was initiated by the addition of CDNB into reaction medium and measured at 340 nm for 2 minutes (ε=0.0096 $\mu M^{-1}cm^{-1}$).

Catalase activities in rainbow trout liver cytosol were determined as described by Aebi (1984) with some modifications. Rainbow trout liver cytosols were pretreated with 1% Triton X-100 for 10 minutes. A typical reaction mixture contained 50 mM H_2O_2. The reaction was initiated by the addition of H_2O_2 and measured at 240 nm for 1 minute (ε= 0.0364 mM^{-1} cm^{-1}).

Glutathione reductase (GR) activities in rainbow trout liver cytosol were determined as described by Carlberg & Mannervick (1985) with some modifications. A typical reaction medium contained 0.5 mM EDTA, pH 7.0, 100 mM potassium phosphate buffer, pH 7.0, 0.1 mM NADPH, 1 mM GSSG, 50 µL of enzyme source, and distilled water at a final volume of 2 mL. The reaction was initiated with the addition of GSSG. The decrease in NADPH amount was measured at 340 nm for 5 min spectrophotometrically (ε = 6.22 $mM^{-1}cm^{-1}$).

The *in vitro* effect of malachite was studied by adding different concentrations of malachite green (2.5-20.0 µM) before the addition of 7-ethoxyresorufin into the reaction mixture of EROD. The composition of reaction mixture was the same as in a typical EROD activity measurement. Microsomes from the control group were used in these activity measurements. The activities obtained at different concentrations of malachite green were compared with the control activity.

Statistical Analysis

In vivo enzyme activity results were expressed as average activity ± standard error of mean (SEM). All data were first tested for normality. The treatment groups were compared with the control group by using an independent samples t-test. The analyses were done by using SPSS statistical package (Version 21.0, Chicago, IL). Differences between means were considered significant when p≤0.05. *In vitro* enzyme activity results were given as percentage of enzyme activity ± standard deviation (SD).

Results

In this study, rainbow trout samples were divided into three groups and treated with two different doses of malachite green (0.1 and 0.5 mg/L). The treatment period for high dose (0.5 mg/L) was ended at 24th hour since five fish samples died. These fish were discarded, and the remaining fish were used for the activity measurements in this group. The treatment period for low dose (0.1 mg/L) was ended at 54th hour. Microsomes and cytosols were prepared from each liver samples. EROD, PROD, ERND, GST, CAT, and GR activities were measured, and the results of each treatment group were compared with those of the control group. All activities were measured in duplicates and, in some cases, in triplicates.

7-ethoxyresorufin O-deethylase (EROD) activities were 13.66 ± 1.24 pmole/min/mg protein in the control group, 12.99 ± 1.78 pmole/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 44.40 ± 7.00 pmole/min/mg protein in the 0.5 mg/L concentration malachite green treated group. EROD activities of fish treated with 0.5 mg/L malachite green were significantly higher than those of the control group (p≤0.05) (Fig. 1A).

7-Pentoxyresorufin O-depentylase activities (PROD) were 0.31 ± 0.02 pmole/min/mg protein in the control group, 0.40 ± 0.05 pmole/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 0.53 ± 0.01 pmole/min/mg protein in the 0.5 mg/L concentration malachite green treated group. PROD activities of fish in the 0.5 mg/L malachite green treated group were higher than those of fish in the 0.1 mg/L malachite green treated group and the control group. PROD activities of fish treated with 0.5 mg/L malachite green were significantly higher than those of the control group (p≤0.05) (Fig. 1B).

Erythromycin N-demethylase (ERND) activities were 91.63 ± 16.33 pmol/min/mg protein in the control group, 124.92 ± 11.34 pmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 115.69 ± 30.25 pmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. ERND activities of treatment groups were slightly higher than those of the control group. However, there were no statistically significant differences in ERND activities between the treatment groups and the control group (Fig. 1C).

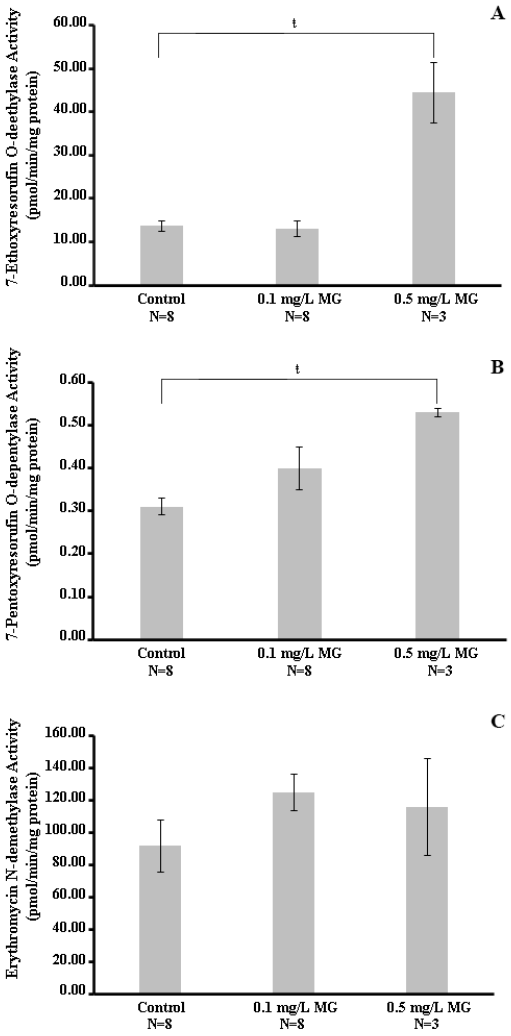

Figure 1 Effect of malachite green on cytochrome P450s. CYP1A-associated 7-ethoxyresorufin-O-deethylase activities (A), CYP2B-associated 7-pentoxyresorufin O-

dependylase (B) and CYP3A-associated erythromycin N-demethylase (C) The symbol (ŧ) indicates statistically significant difference between the 0.5 mg/L malachite green treated group and the control group (p≤0.05).

Glutathione S-transferase activities (GST) were 1162 ± 95 nmol/min/mg protein in the control group, 1109 ± 36 nmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 1165 ± 67 nmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. When the groups were compared, no statistically significant difference was found between the treatment groups and the control group (Fig. 2A).

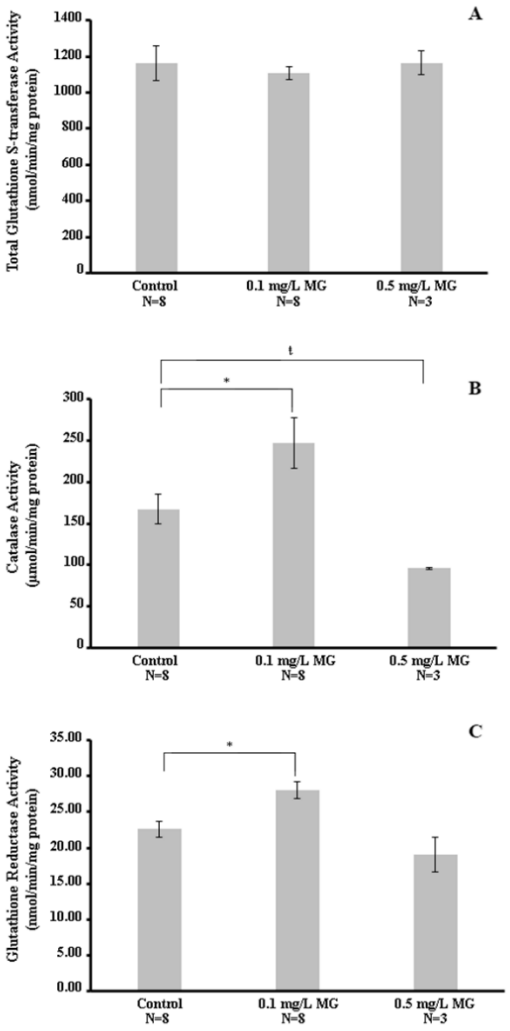

Figure 2 Effect of malachite green on glutathione S-transferase and antioxidant system. Glutathione S-transferase (A), catalase (B) and glutathione reductase (C) The symbol (*) indicates the significant difference between the 0.1 mg/L malachite green treated group and the control group (p≤0.05). The symbol (ŧ) indicates the significant difference between the 0.5 mg/L malachite green treated group and the control group (p≤0.05).

Catalase (CAT) activities were 167 ± 18 µmol/min/mg protein in the control group, 247 ± 31 µmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 96 ± 1 µmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. Catalase activities of rainbow trout samples treated with 0.1 mg/L malachite green were significantly higher than those of the control group (p≤0.05) (Fig. 2B). Catalase activities of fish treated with 0.5 mg/L malachite green were significantly lower than those of the control group (p≤0.05).

Glutathione reductase activities (GR) were 22.58 ± 1.09 nmol/min/mg protein in the control group, 28.05 ± 1.17 nmol/min/mg protein in the 0.1 mg/L concentration malachite green treated group, and 19.08 ± 2.40 nmol/min/mg protein in the 0.5 mg/L concentration malachite green treated group. GR activities of fish treated with 0.1 malachite green were significantly different from those of the control group (*p*≤.05) (Fig. 2C).

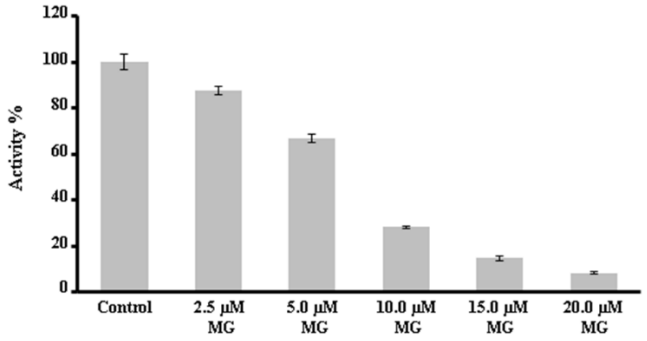

Figure 3 Inhibitory effect of malachite green on CYP1A-associated 7-ethoxyresorufin-O-deethylase activity.

In addition to *in vivo* studies, the effect of malachite green on EROD activities was determined by *in vitro* studies. As the concentration of malachite green increased in the reaction mixtures, EROD activities decreased (Fig. 3). At 20 µM concentration of malachite green, only 8 % of the initial EROD activity remained.Discussion

The use of malachite green has not been permitted in many countries. However, the monitoring studies clearly indicate its illegal usage (Hakami et al., 2021; Gharavi-nakhjavani et al., 2023; Lemos et al., 2023). High amounts of malachite green have also been reported in several effluents (Khan et al., 2019). The concentration of malachite green has been determined as 1.320 mg/L in

laundry effluent, 0.620 mg/L in paper effluent, 0.790 mg/L in printing effluent, and 1.680 mg/L in textile effluent (Khan et al., 2019). *In vivo* researches on its effect and metabolism in fish is limited. In the current study, 62% of fish samples died in the 0.5 mg/L malachite green treatment group within the 24 hours of malachite green administration. This result clearly shows that fish may die if exposed to the malachite green amounts present in these effluents. Considering the amount of accumulation and mortality at the dose of 0.5 mg/L, we ended the chemical administration period of the 0.1 mg/L malachite green treatment group at the end of 54 hours. Cytosols and microsomes were prepared from all samples. Cytochrome P450 associated enzyme activities and antioxidant enzyme activities were measured in microsomes and cytosols, respectively.

Cytochrome P450s metabolize both endogenous and exogenous molecules. Therefore, it is important to determine the effect of malachite green on cytochrome P450s. It has been shown that CYP1A-associated arylhydrocarbon hydroxylase activity increases with malachite green treatment in SHE primary cultures (Panandiker et al., 1992). The results of an *in vitro* study report that CYP1A-associated EROD activity decreases with the addition of increasing concentrations of malachite green into the reaction medium (Nebbia et al., 2017). However, there are contradictory reports about the effect of malachite green on CYP1A. For example, CYP1A has been measured in mRNA level in carp. Its level decreases with 0.146 mg/L chronic administration of malachite green (Sinha et al., 2021). Similarly, the mRNA expression level of CYP1A was inhibited significantly by the administration of 0.5 mg/L malachite green in Nile tilapia, grass carp, and Taiwan snakehead (Li et al., 2013). In the current study, elevated EROD activities were measured in the 0.5 mg/L malachite green treatment group. CYP1A activity is involved and stimulated by malachite green. Our *in vitro* studies also supported the involvement of CYP1A in the biotransformation of malachite green in rainbow trout (Fig. 3). Other cytochrome P450 associated activities measured in this study were ERND and PROD activities. CYP3A associated ERND activities were higher in the treatment groups. However, these results were not statistically different from the control group, indicating that this activity is not affected by the acute malachite green treatment. The PROD activity is generally associated with CYP2B enzyme activity in humans (Lubet et al., 1985). In the current study, this activity was higher in the treatment groups. Significantly high PROD activities were measured in the 0.5 mg/L malachite green treated group than in the control ($p \leq 0.05$). These findings suggest that cytochrome P450 enzymes associated with EROD and PROD activities are involved in the metabolism of malachite green.

Phase II enzymes also play important roles in the biotransformation of xenobiotics. Glutathione S-transferases are phase II enzymes catalyzing the conjugation reactions of many xenobiotics. In this study, GST activity in the 0.1 mg/L malachite green administration group was lower than that in the control group. However, this difference was not significant. A similar decrease in GST activity has been reported in carp (Sinha et al., 2021). In another study, administration of 0.1 mg malachite green for 30 days reduced GST activity in swiss albino mice (Das et al., 2013). Similarly, the mRNA expression level of GST was significantly inhibited by the administration of 0.5 mg/L malachite green in Nile tilapia, grass carp, and Taiwan snakehead (Li et al., 2013). The involvement of GST in the biotransformation of malachite green has been shown with *in vitro* inhibition studies (Nebbia et al., 2017). However, the results of the current *in vivo* study clearly show that this activity was not modulated by acute treatment of malachite green. The insignificant decrease in GST activity in this study may have potentially resulted from the depletion of GSH. It has been shown that GSH level decreases with malachite green treatment in common carp and rainbow trout (Yonar & Yonar, 2010; Sinha et al., 2021).

In addition, studies indicate that malachite green causes free radical formation (ROS) and oxidative stress (Kovacic & Somanathan, 2014). In this study, catalase and glutathione reductase activities were measured to determine the effect of malachite green on antioxidant enzyme activities in the cytosols of rainbow trout. Catalase activity was significantly higher in the 0.1 mg/L malachite green treatment group than the control group, whereas this activity was significantly lower in the 0.5 mg/L malachite green administration group than the control group ($p \leq 0.05$). Similarly, it has been reported that 0.146 mg/L malachite green administration increases catalase activity when carp samples are treated with malachite green for 15 days (Sinha et al., 2021). In the same study, catalase activity decreased in the 30- and 60-day administration groups. In the current study, the reason for low activities measured in 0.5 mg/L malachite green treated group might result from high malachite green administration in a short period of time. A similar decrease in catalase activity has been reported in the liver of rainbow trout treated with 6.67 mg/L malachite green (Yonar & Yonar, 2010). Significantly higher glutathione reductase activities were measured in the 0.1 mg/L malachite green administration group compared with the control group ($p \leq 0.05$). These results indicate that glutathione reductase

activity was also modulated by the treatment of malachite green. The modulation patterns of catalase and glutathione reductase activities were similar, higher in the low dose malachite green administration group than in the control and lower in the high dose malachite green administration group. Both activities are related to the elimination of reactive oxygen species from the body. The results of this study clearly show that malachite green affects oxidative stress defense system in rainbow trout.

Conclusion and Recommendations

As a result of this study, CYP1A-associated activity increased in the high malachite green administration group. Antioxidant enzyme activities were also modified by malachite green treatment. CYP1A is generally found in the conversion of toxic chemicals to more toxic forms and generation of reactive oxygen species. The high mortality observed in the current study at 0.5 mg/L may be related to generation of excess oxygenated cytochrome P450 related biotransformation products and reactive oxygen species. Further studies are needed to determine whether the high mortality rate in the high malachite green administration group is directly caused by malachite green or by other factors related to modified enzyme activities.

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