



Toxic Effects of Glyphosate-Based Herbicide on *Melanopsis praemorsa*

Özlem DEMİRÇİ^{1*}, Feysel ÇAKMAK², Ahmet İsmail ÖZKAN³

¹Dicle University, Science Faculty, Biology Department, Diyarbakır, Turkey
ozdem22@gmail.com, ORCID: 0000-0001-9511-2010

²Dicle University, Science Faculty, Molecular Biology and Genetics Department, Diyarbakır, Turkey
feyselcakmak@gmail.com, ORCID: 0000-0002-4827-150X

³Dicle University, Science Faculty, Molecular Biology and Genetics Department, Diyarbakır, Turkey
aiozkan.13801509@ogr.dicle.edu.tr, ORCID: 0000-0002-4511-2386

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Abstract

In the present study, we determined the 24, 48, 72, and 96 h LC₅₀ values for a glyphosate-based commercial herbicide (GBH) for the aquatic snail *Melanopsis praemorsa*. We examined subacute toxicity responses from *M. praemorsa* after 30-day exposure to 1/10, 1/5, and 1/2 of the 96 h LC₅₀ value using several biochemical markers. Glutathione reductase (GR) and glutathione S-transferase (GST) activities were significantly inhibited when compared to control after 4 days of GBH exposure. On the twentieth day of exposure, GST and GR activities were inhibited in organisms exposed to both LC₅₀/10 and LC₅₀/5 compared to control. These results showed that chronic GBH exposure inhibited GST, an important detoxifying enzyme. GR, an important oxidative stress marker, was likely inhibited as a result of inhibition of the detoxification mechanism.

Keywords: *Melanopsis praemorsa*; Biomarkers; Glyphosate-based herbicide.

Glifosat Bazlı Herbisitinin *Melanopsis praemorsa* Üzerindeki Toksik Etkileri

Öz



Bu çalışmada, sucul salyangoz *Melanopsis praemorsa* için glifosat bazlı bir ticari herbisit (GBH)'in 24, 48, 72 ve 96 saat LC₅₀ değerlerini belirledik. *M. praemorsa*'nın kronik toksisite tepkilerini ve 96 saat LC₅₀ değerinin 1/10, 1/5 ve 1/2'sine 30 gün maruz kaldıktan sonra *M. praemorsa*'nın kronik toksisite cevaplarını birkaç biyokimyasal belirteç kullanarak inceledik. Glutasyon redüktaz (GR) ve glutasyon S-transferaz (GST) aktiviteleri, 4 günlük GBH maruziyetinden sonra kontrol ile karşılaştırıldığında istatistiksel olarak anlamlı şekilde inhibe oldu. Yirmi günlük maruz kalma sonucunda, GST ve GR aktiviteleri kontrole karşılaştırıldığında hem LC₅₀/10'a hem de LC₅₀/5'e maruz kalan organizmalarda inhibe olmuştur. Bu sonuçlar, kronik GBH maruziyetinin önemli bir detoksifiye edici enzim olan GST'yi inhibe ettiğini gösterdi. Önemli bir oksidatif stres belirteci olan GR, detoksifikasyon mekanizmasının inhibisyonu sonucu inhibe edildiği sonucuna varılabilir.

Anahtar Kelimeler: *Melanopsis praemorsa*; Biyobelirteçler; Glifosat bazlı herbisit.

1. Introduction

Pesticides are xenobiotics that routinely contaminate aquatic areas such as rivers, lakes, and coastal areas [1]. Agricultural area development has markedly increased pesticide use. Pollution of aquatic environments by pesticides usually occurs by surface waters and subsurface drainage after intensive agricultural applications. Pesticides that reach water resources often cause striking toxicity to non-target organisms. Therefore, the evaluation of pesticide effects on aquatic vertebrates and invertebrates is very important [2]. Acute and chronic toxicity tests are frequently used to determine the potential effects of xenobiotics on aquatic populations [3].

Glyphosate has been one of the most widely-used ingredients worldwide since it was introduced to the market in the 1970s. The most important reason for this use is that glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway. It functions as a non-selective herbicide to inhibit the synthesis of aromatic acids and many different aromatic compounds in plants, algae, bacteria, and fungi [4, 5]. While this pathway is absent in animals, many studies have shown that glyphosate is toxic to non-target aquatic organisms [6-8]. Due to its high solubility in water and extensive use, exposure of non-target aquatic organisms to this herbicide has caused great concern [9].

Invertebrates are organisms that are widely distributed, represented by many species, and occupy lower levels of the trophic chain. Many bioassays that use aquatic invertebrates have shown that they are more sensitive to xenobiotics than aquatic vertebrates [10]. Many xenobiotics are transferred from invertebrates to vertebrates through accumulation by nutrition. For these

reasons, aquatic invertebrate toxicity testing is a very useful tool for performing the “3Rs principle” (replace, reduce, and refine) in experiments to investigate the safe use of many chemicals [11]. *Melanopsis praemorsa* (Linnaeus, 1758, Buccinum), a gastropod snail, was chosen for this study because it is the most abundant species in the Mediterranean region and exhibits several characteristics: widespread in freshwater, available in all seasons, and able to adapt to laboratory conditions.

Pesticides can accumulate in aquatic organisms and can thus reach humans as the last step in the food chain [2, 12]. Some recent studies have shown that pesticides promote oxidative stress by causing an increase in reactive oxygen species (ROS) [13, 14]. Live systems have enzymatic and nonenzymatic antioxidant systems to ameliorate ROS, and their responses are very useful biomarkers for revealing potential toxicity [15]. The antioxidant protection system contains important enzymes such as glutathione reductase (GR) and catalase (CAT). GR catalyses the NADPH-dependent conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the glutathione cycle. GSH is used in hydrogen peroxide (H_2O_2) and organic peroxide detoxification by glutathione peroxidase (GPx) [16]. Without this process, H_2O_2 can be converted to the high reaction hydroxyl radical ($\cdot OH$) through the Haber-Weiss or Fenton reactions [17, 18]. Glutathione *S*-transferase (GST), an important phase II metabolism enzyme that is part of detoxification mechanisms, can be used as a biomarker to reveal pollutant effects on organisms [19].

Glyphosate-based herbicides (GBHs; e.g., Roundup®) are widely used in aquatic and terrestrial environments. The effects on aquatic organisms such as plants, green algae, fishes, amphibians, and invertebrates have been investigated, and attempts have been made to overcome GBH toxicity in aquatic environments [20-22]. However, studies on snails, an important member of the aquatic ecosystem, are very limited in the literature. The aim of this study was to investigate the biochemical aspects of *M. praemorsa* as bioindicators for realistic evaluation of the effects of GBHs on the aquatic environment.

2. Materials and Methods

2.1. Chemicals

In this study, the effects of a commercial formulation of an herbicide (Roundup®) that contained glyphosate isopropylammonium salt (360 g L^{-1}) as an active ingredient and polyethoxylamine amine as a surfactant were examined. Roundup® was supplied from a local agricultural supply store. The chemicals used for biochemical analyses were obtained from Sigma-Aldrich Chemical Company (USA).

2.2. Acute toxicity assay

The median lethal concentration that caused 50% mortality (LC_{50}) was determined using the semi-static renewal acute toxicity test [23]. Experiments were carried out with 3 replicates and 10 individuals in 5 L glass containers. Dechlorinated tap water was used in all experiments. Organisms were not fed during the acute toxicity test period. Range determination experiments were performed prior to the actual experiment to determine the GBH LC_{50} for the snail. The GBH exposure concentrations were 0.1, 0.3, 0.9, 2.7, 8.1, 24.3, 72.9, 281.7, 656.1, and 1968.3 μg active ingredient (AI) L^{-1} . Inactivity of pesticide-exposed individuals was regarded as evidence of death, at which time dead individuals were removed from the glass containers.

2.3. Test animals and experimental design

Individual *M. praemorsa* (10.1 ± 2 mm in length and 402 ± 4 mg in weight) were obtained from a non-polluted freshwater area on the Dicle University campus ($36^{\circ}54'56.84''\text{N}$ $40^{\circ}16'28.50''\text{E}$) at April 2018. Some physicochemical properties of the field were: oxygen amount was 7.40 mL^{-1} , the temperature was 19.4°C , and pH was 7.04. The concentration of dissolved oxygen in the environment was 7.08, the temperature was 22°C and the pH value was 8.01 during acclimatization and experiments. All experiments were performed at under a 13h:11 h light: dark cycle and the subjects were fed *ad libitum* with *Lactuca sativa* leaves every 24 h to adapt to laboratory conditions. Experiments were carried out in 5 L glass containers under semi-static conditions and with 3 replicates. The experimental setup was prepared in such a way that 30 individuals were at each glass aquaria. Organisms were exposed to the $LC_{50}/10$, $LC_{50}/5$ $LC_{50}/2$ GBH (Roundup®) concentrations for 96 h. No deaths were observed during the experiment. On days 4, 10, 20, and 30, the organisms were sacrificed, washed with distilled water, and stored at -80°C for enzyme analyses.

2.4. Enzyme analyses

The organisms stored at -80°C were weighed as a whole and homogenized using four volumes of homogenization phosphate buffer (pH 7.4) under ice condition. Then, samples were centrifuged at 20,000 g for 15 min at 4°C and the supernatant was assayed for enzyme activities. GST and GR enzyme activities were measured using a microplate reader system [8, 24, 25]. The total protein concentration of each sample was measured according to the Bradford method using bovine serum albumin as a standard [26].

2.5. Statistical analysis

24, 48, 72, and 96 h LC₅₀ values were calculated using Probit Analysis [27]. The SPSS statistical software package (version 15.0) was used to statistically evaluate enzyme activities. One-Way ANOVA test was used to determine differences between the control and treatment groups and to demonstrate any dose-dependent changes. The Mann-Whitney *U*-test was used to determine the statistical significance of pairwise comparisons. For all analyses, $p < 0.05$ was considered significant.

3. Results

We first determined the effects of different concentrations of GBH on *M. praemorsa*. The LC₅₀ values of GBH were determined for 24, 48, 72 and 96 h exposure as 21.8, 12.6, 10.6 and 9.3 mg L⁻¹, respectively (Table 1). The 96 h LC₅₀ concentration was used for subsequent studies to determine changes in GR and GST activities.

GST activity in organisms exposed to both LC₅₀/5 and LC₅₀/2 concentrations on day 4 of the application was significantly inhibited when compared to control and (Table 2). On day 10 of the application, GST activity in organisms exposed to the LC₅₀/2 concentration was significantly inhibited compared to the LC₅₀/5 group. After 20 days of exposure, GST enzyme activity in the organisms exposed to all concentrations was different from the control; enzyme activity was decreased at the lowest concentration while it was induced in a concentration dependent manner compared to control, LC₅₀/5 and LC₅₀/10 groups. GST enzyme activity in individuals exposed to both LC₅₀/10 and concentration after 30 days was significantly increased compared to the control.

After 4 days of exposure, the GR activity in LC₅₀/2 and LC₅₀/5 groups was decreased compared to the control; enzyme activity at the lowest concentration (LC₅₀/10) was significantly higher than the other concentrations (Table 2). However, no statistically significant difference was observed between any groups after 10 days. GR activity decreased after 20 d of exposure while the most significant decrease was observed at the lowest concentration. After 30 days, only the individuals exposed to the LC₅₀/5 concentration exhibited higher GR activity compared to control (Table 2).

Table 1: The 24 h, 48 h, 72 h and 96 h LC₅₀ values of glyphosate-based herbicide (GBH) on *Melanopsis praemorsa*

	Test range (AI)	No tested	LC ₅₀			
			24 h	48h	72h	96 h
GBH(mg L ⁻¹)	1.0-50.0	30	21.7	12.6	10.5	9.3

Table 2: *Melanopsis praemorsa* GST and GR activities during 30 days GBH exposure

Exposure period (Day)	Concentration	GST	GR
4 d	Control	3.07 ± 0.46	3.91 ± 0.18
	LC ₅₀ /10	1.83 ± 0.05	3.19 ± 0.25 ^{b,c}
	LC ₅₀ /5	1.85 ± 0.50*	1.78 ± 0.46 ^{*,a}
	LC ₅₀ /2	2.78 ± 0.44*	1.77 ± 0.05 ^{*,a}
10 d	Control	2.54 ± 0.73	2.19 ± 0.32
	LC ₅₀ /10	2.37 ± 0.44	1.57 ± 0.42
	LC ₅₀ /5	2.85 ± 0.26 ^c	2.19 ± 0.27
	LC ₅₀ /2	1.30 ± 0.20 ^b	2.03 ± 0.20
20 d	Control	2.70 ± 0.12	3.96 ± 0.30
	LC ₅₀ /10	1.18 ± 0.13 ^{*,b,c}	2.03 ± 0.21 ^{*,b,c}
	LC ₅₀ /5	2.01 ± 0.21 ^{*,a,c}	3.24 ± 0.18 ^{*,a}
	LC ₅₀ /2	3.37 ± 0.24 ^{*,a,b}	3.28 ± 0.27 ^a
30 d	Control	1.02 ± 0.27	1.50 ± 0.30
	LC ₅₀ /10	1.77 ± 0.17*	2.03 ± 0.21
	LC ₅₀ /5	1.72 ± 0.32	2.31 ± 0.25*
	LC ₅₀ /2	1.34 ± 0.22	1.98 ± 0.23

Enzyme activities are expressed as nmol/min/mg protein ± standard error ($N=3$).

* $p < 0.05$ compared to control.

^a $p < 0.05$ compared to LC₅₀/10.

^b $p < 0.05$ compared to LC₅₀/5.

^c $p < 0.05$ compared to LC₅₀/2.

4. Discussion

Glyphosate is a broad-spectrum herbicide and currently the most widely used herbicide worldwide. It is used for over 750 different products in agriculture, forestry, and domestic applications. GBHs have begun to be used more intensively and in much wider areas, especially with the increase of glyphosate-resistant genetically-modified products [24]. Glyphosate residues have been found in the air, aquatic environments, and food. Thus, GBHs represent a major threat to the aquatic ecosystem, and the search for effects on non-target aquatic organisms, such as in the present study, will allow a greater understanding of its toxic potential.

The past and present use of many pesticides in agricultural and urban areas has led to their presence in surface and underground water resources [28-31]. Roundup® and other glyphosate-based formulations are used extensively in agricultural areas as well as for weed control in aquatic

areas [32]. Numerous aquatic toxicity studies have been performed with GBHs [33-35]. The toxic effects of the active ingredient (glyphosate) and commercial formulations have been previously compared. These reports revealed that the commercial GBH formulations caused more toxic effects compared to glyphosate alone [36-38]. However, effluents are always contaminated with commercial pesticides. Therefore, investigation of the effect of commercial pesticides on non-target organisms can provide very realistic reports [39]. Many bioassays with aquatic organisms have shown that invertebrates are generally more sensitive to xenobiotics than vertebrates [10]. In particular, gastropods, the most abundant mollusc group, are important bioindicators for many different contaminants such as metals and pesticides [40]. Thus, this study investigated changes in GR and GST enzymes, very important biochemical markers for GBHs, on *M. praemorsa*, a gastropod species with a wide distribution in the Mediterranean.

In our study, we determined the 24, 48, 72 and 96 hours LC_{50} values of GBH for *M. praemorsa* as 21.8, 12.6, 10.6 and 9.3 mg L⁻¹, respectively. In our study, we determined the 24, 48, 72 and 96 hours LC_{50} values of GBH for *M. praemorsa* as 21.8, 12.6, 10.6 and 9.3 mg L⁻¹, respectively. In studies using commercial formulations of Glyphosate, LC_{50} values similar to our results were determined. Bakry et al. [41], Abdel-Ghaffar et al. [42] and Omran et al. [43] determined the 24 h LC_{50} value for *Biomphalaria alexandrina* to be 3.15, 15.062, 41.6 mg L⁻¹, respectively. Different commercial forms of glyphosate were used in these three studies. However, the LC_{50} value is quite different in studies where the active ingredient (AI) of glyphosate is used. For example, Xu et al. [35] 24, 48, 72, 96 h LC_{50} values for freshwater snail (*Pomacea canaliculata*) exposed to glyphosate AI was determined as 178.2, 176.5, 176.2 and 175.1 mg L⁻¹, respectively.

GR activity decreased in the treatment groups (except on exposure days 10 and 30) compared to control. GR is a reliable biomarker that is widely used in aquatic toxicology assessments [44-46]. In some previous studies, GR activity was inhibited in aquatic organisms exposed to pro-oxidants [47-49]. There are two types of glutathione in organisms, GSSG and GSH, and the GSH:GSSG ratio in normal cells is greater than 100:1 [50]. The amount of GSH in tissues usually decreases with short-term oxidant exposure but increases after prolonged exposures. Reduction in GSH levels causes the organs to be more sensitive to xenobiotics. In several studies, moderate oxidative stress increased GSH levels [51]. GR maintains the sulfhydryl groups of both cytosolic proteins in the reduced state by allowing the GSH reserve in cells to remain at the correct level and contributes to the detoxification of many pro-oxidant xenobiotics [3]. Our results revealed that in *M. praemorsa* GR was inhibited compared to control, especially after 4- and 20-day GBH exposure. Contrary to our results, Barky et al. [41] showed a significant

increase in GR activity of freshwater snails (*B. alexandrina*) exposed to GBH. However, in another study done by Bakry et al., [52] similar to our study results, they found that GR activity of a different freshwater snail (*Bulinus truncatus*) exposed to GBH was significantly inhibited. Similar to the GR enzyme in our study, it was found that *M. praemorsa*, exposed to 4 and 20 days GBH, was inhibited compared to the GST enzyme control. GST activity was only induced in organisms exposed to the highest concentration (LC₅₀/2) after 20 days compared to control. These results may suggest that GST inhibition is due to an inability to maintain an adequate GSH level or due to GR enzyme inhibition.

In phase II detoxification, GST catalyses the conjugation of xenobiotics with GSH to aid its removal from the organism [53]. Xenobiotics may cause cellular damage. Therefore, cytosolic GST induction is used as a biomarker for environmental pollutants [54, 55]. Many studies demonstrated that GST activity was induced in aquatic organisms exposed to pesticides [5, 48, 56]. Similarly, Khalil [57] reported that GST activity in the freshwater snail *Lanistes carinatus* exposed to chlorpyrifos for 28 days was increased compared to control. However, Lushchak et al. [58] reported that GST and GR enzyme activities in *Carassius auratus* tissues exposed to GBH were generally suppressed. In another study, liver GST activity was inhibited in two different fish species (*Anabas testudineus* (Bloch) and *Heteropneustes fossilis* (Bloch)) after GBH exposure [59]. Similarly, we found that *M. praemorsa* GST activity was generally inhibited after GBH exposure compared to control. The inhibition of detoxification enzymes, when considered independently from reduced GR activity, could make xenobiotics more toxic to an organism and cause metabolic dysfunction [55]. These results provide inexorable evidence that the detoxification mechanism can be inhibited by the GBH.

5. Conclusions

Since glyphosate is a non-selective herbicide, it is used in many different terrestrial and aquatic areas against undesirable vegetation. Thus, glyphosate is one of the most widely used herbicides globally. There are many studies that have investigated its toxic effects, especially for aquatic organisms. The effects of GBHs on the aquatic snail may provide an important contribution to the proper assessment of their aquatic toxicity.

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