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# Toxicity of Paraquat and Dicamba on Caenorhabditis Elegans LC50 Value

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**ABSTRACT**

Paraquat and dicamba are chemicals commonly used in agriculture for plant control. The US Environmental Protection Agency (EPA) has classified paraquat into a restricted use class for use only by practitioners, as it is highly toxic. In this study, the effects of different concentrations of paraquat and dicamba toxic substances on *C. elegans* were studied. In tests, *C. elegans* were directly exposed to different concentrations of paraquat and dicamba for 6h, 12h, 18h, 24h. In particular, it was determined at which paraquat and dicamba doses that half of the *C. elegans* individuals (LC50) died. In the analysis results, paraquat LC50 values were found as LC50 6h= $LC_{50\ 6h}$= 7412 µM, $LC_{50\ 12h}$= 459 µM, $LC_{50\ 18h}$= 123 µM, $LC_{50\ 24h}$= 61 µM. Similarly, dicamba LC50 values were found as $LC_{50\ 6h}$= 14610 µM, $LC_{50\ 12h}$= 1404 µM, $LC_{50\ 18h}$= 906 µM, $LC_{50\ 24h}$= 463 µM.

***Keywords:*** C. elegans, Paraquat, Dicamba, Lethal concentration (LC), Paraquat and dicamba toxicity.

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## Introduction

Paraquat (1,1'-dimethyl-4-4'-bipyridinium dichloride, PQ) is a fast, effective and non-selective highly toxic dipyridyl herbicide widely used especially in developing countries [1,2]. Paraquat toxicity is mediated by oxidative stress-induced mechanisms [3]. Paraquat exerts its effectiveness by interfering with intracellular electron transfer photosystems by inhibiting the reduction of oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) to nicotinamide adenine dinucleotide phosphate (NADPH) during photosynthesis. Instead of NADPH, the paraquat ion is reduced and a monocation-free radical is formed. The electron transferred to the paraquat is then rapidly converted to oxygen with the production of superoxide, leading to the formation of reactive oxygen species (ROS) such as superoxide anion ($O_2^-$), singlet oxygen ($^1O_2$), peroxyl radicals (ROO-) [4]. As a result, oxidative damage occurs due to reactive oxygen species [5-7]. Mitochondrial dysfunction results in decreased cellular energy supply, inability to maintain cellular homeostasis, and activation of cell death [8,9]. At the same time, paraquat causes respiratory depression through inhibition of mitochondrial complexes [10]. Paraquat also has acute systemic effects. In particular, the lungs and kidneys are the organs most susceptible to paraquat-induced injuries [11]. An acceptable daily intake (ADI) of 0.004 mg/kg has been established for Paraquat. Also, the minimum lethal dose of paraquat in humans is about 35 mg/kg body weight [12]. In case of exposure to lethal doses of paraquat, death can occur within a few days after inhalation [13]. Paraquat (PQ) has been associated with Parkinson's (PD) in epidemiological studies in rodents and it has been reported that paraquat is an environmental risk factor for PD disease [14]. In particular, paraquat was thought to cause parkinsonism by increasing oxidative stress and affecting a pathophysiological genetic mechanism (alpha-synuclein, PINK-1, DJ-1 and PARKIN mutations) [15,16]. However, the evidence that human exposure to chemicals poses an increased risk for PD is quite limited and is based on insufficient epidemiological data [17].

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is a white solid crystalline compound with a molecular formula ($C_8H_6Cl_2O_3$) and a molecular weight of 221.03 g/mol. Dicamba is a chlorinated derivative of o-anisic acid, first registered in the United States in 1967. O-Anisic acid, (ortho-methoxybenzoic acid or 2-methoxybenzoic acid) is a crystalline solid and is an isomer of anisic acid [18]. The biodegradation metabolites of Dicamba are 2,5-dichlorophenol and 3,6-dichlorosalicylic acid. After biodegradation, 3,6-dichlorosalicylic acid converts to more carbon dioxide, water and chloride [19]. These compounds can be dangerous if not handled properly, and many (even though not all of them) pose a potential hazard to the environment due to contamination of food, water and air [20]. In addition, hazardous anthropogenic activities continuously release large amounts of these compounds into the environment, regardless of their bioaccumulation and toxicity. However, it is well known that this chemical not only affects target organisms, but also exerts adverse effects on non-target organisms [21]. Dicamba has been detected as a contaminant in agricultural, urban and mixed agriculture/urban areas, surface drinking water reservoirs and even estuarine waters [22]. Much is known about the effects of dicamba

on aquatic plants and its risks to terrestrial organisms. So far, the potential genotoxic hazard of dicamba has been demonstrated by various in vivo and in vitro tests [23]. Some recent research has shown that dicamba should be viewed as a DNA-damaging agent. Similarly, studies have reported that dicamba causes induction of sister chromatid exchanges (SCEs) and mitotic changes in human lymphocytes and Chinese hamster ovary (CHO-K1) cells [24]. Although the information on the potential toxic effects of dicamba is limited, it is known how unsaturated membrane fatty acids affect cis-trans isomerization, toxicity and membrane activity, and the toxicity of dicamba increases with the increase in the length of the alkyl chain lengths of dicamba [25].

The nematode *Caenorhabditis elegans* is frequently preferred in biological research due to its convenient use in intergenerational studies, short breeding period and low cost. Studies have revealed that *C. elegans* contains many genes homologous to mammals [26]. Moreover, the similarity of *C. elegans* biological processes with human processes has made *C. elegans* a useful model for genetic and developmental biological research [27]. In addition, the clear traceability of genetic and biochemical processes of *C. elegans* provides the opportunity to use this organism as a biosensor for toxicological studies [28]. For these reasons, many pharmaceutical companies use the positive predictive power of *C. elegans* in their drug development processes and chemical toxicity tests [29,30].

In this study, the effects of different concentrations of paraquat and dicamba toxic substances on *C. elegans* were studied. In particular, it was determined at which paraquat and dicamba doses that half of the *C. elegans* individuals ($LC_{50}$) died.

## Materials and Methods

### Collection of Materials

In this study, it was aimed to investigate the toxicological effects of analytically pure dicamba and paraquat chemicals on Caenorhabditis elegans. Dicamba was stored at room temperature in the dark until use, while Paraquat was stored at +4 °C. In the study conducted to examine the toxicological effects of dicamba and paraquat chemicals, living organisms (wild type C. elegans N2) and bacterial colonies (*E. coli* OP50 strain) were provided by the University of Minnesota *Caenorhabditis* Genetics Center (CGC).

### Preparation of TBX Agar Medium and Purification of E. coli OP50

Tryptone Bile X-glucuronide (TBX) Agar medium was preferred to determine the presence of *E. coli* OP50 strain in cultures and to purify the strains. 3.65 g of TBX Agar required to prepare 100 mL of medium was weighed and prepared in $dH_2O$. The prepared medium solution is kept at 125°C for 15 minutes. After autoclaving, it was cooled (55 °C) and poured into 60 mm petri dishes as 10 mL. Solidified TBX Agar media were seeded from the stock *E. coli* OP50 strain. The inoculated petri dishes were incubated for 24 hours at 37 °C and the blue colonies formed were determined.

### Preparation of LST Broth and Propagation of E. coli OP50

Lauryl Sulfate Broth (LST) broth was used to propagate *E. coli* OP50 strain. The medium was prepared to contain 9.125 g of LST in 250 ml of $dH_2O$. 15 min at 125°C. Pure *E. coli* Op50 colonies determined in TBX medium were transferred to the broth that came to 37 °C after autoclaving. The medium was kept in an incubator at 37 °C for 24 hours. The stock broth containing the *E. coli* OP50 strain determined using the McFarland method was kept in the refrigerator at +4 °C to be used.

### Preparation of Nematode Medium (NGM) and C. elegans Cultures

*C. elegans* can be cultured in laboratories on Nematode Growth Medium (NGM) agar. To prepare NGM; 2.5 g Peptone, 3 g NaCl and 20 g Agar were mixed in 1 L dH2O until boiling point. 15 minutes at 120 ºC. After autoclaving, it was kept in a water bath at 55 ºC. For homogenization of NGMs; 1 mL of $MgSO_4$ (1M), 1 mL of cholesterol (5 mg/mL), 1 mL of $CaCl_2$ (1M), 25 mL of $KPO_4$ buffer (pH:7) were filtered through cellulose filters and added to the medium solution. Approximately 10 mL of homogenized NGMs were poured into 60 mm petri plates. Next, 500 µL of LST medium containing *E. coli* Op50 was placed in the midpoint of NGM. Afterwards, small incisions were taken from the *C. elegans* stock culture medium and placed in the middle of the prepared NGM, and fresh *C. elegans* cultures were created.

### C. elegans Synchronization

An alkaline hypochlorite solution was used to synchronize cultures of *C. elegans*, which dissolves the eggs of hermaphrodites without damaging them [31]. The petri dish was washed by pipetting with 2 ml of $dH_2O$ to loosen the eggs. The liquid was collected in a capped sterile 5 mL conical centrifuge tube and the total volume was made up to 3.5 mL with $dH_2O$. 0.5 mL NaOH (1g/5ml) and 1 mL NaOCl were added into the tube. The tube was shaken every 2 minutes for a total of 10 minutes. To pellet the released eggs, the tube was run at 3400 rpm for 5 minutes centrifuged. The liquid in the tube was aspirated up to 0.25 mL and transferred to a bottle containing 25 mL of M9 buffer (0.3 g $KH_2PO_4$, 0.6 g $Na_2HPO_4$, 0.5 g NaCl, 0.1 mL 1 M $MgSO_4$) and incubated for 1 day at 20°C to obtain highly starved L1 larvae. The flask was then placed on ice for 15 minutes and centrifuged to allow *C. elegans* to settle. After re-aspiration after centrifugation, 0.25 mL of liquid remaining was transferred with a Pasteur pipette to a clean NGM petri dish containing *E. coli* OP50. Thus, *C. elegans* life forms were synchronized by growing at the same time and were used in studies.

### *Preparation of Dicamba and Paraquat Solutions*

The dicamba and paraquat stock solutions used in the study were taken with a micropipette and diluted to a final concentration of 15.625- 31.25- 62.5- 125- 250- 500- 1000 and 2000 µM in the tubes. In the tubes used as control, dicamba and paraquat were not added. *C. elegans* were transferred directly into the prepared solutions.

### *Statistical Analysis*

In the study, SPSS 26.00 probit test was used and $LC_{50}$ values between dose groups were determined. In addition, differences between doses with Anova were determined. In addition, various statistical data including survival time and survival rate using Kaplan-Meier test and Chi-square test are presented together in the findings section. All of the analyzes made in the study were evaluated by taking the averages and graphs and charts were created using the data found.

## Results

### *C. elegans Survival Analyzes*

In the study, 60 *C. elegans* individuals were exposed to 8 different doses of paraquat (15.6- 31.2- 62.5- 125- 250- 500- 1000 and 2000 µM) in tubes for 6,12,18 and 24 hours. After the lowest dose exposure of 6, 12, 18 and 24 hours, 3, 6, 11 and 18 of the *C. elegans* individuals died, respectively. After 6, 12, 18 and 24 hours of exposure at the highest dose, 19, 39, 52 and 56 *C. elegans* individuals died, respectively (Table 1).

Table 1 The effect of paraquat doses on the number of deaths

| Number of individuals | Concentration (µM) | Mortality (number of dead individuals) | | | |
|---|---|---|---|---|---|
| | | 6 h | 12 h | 18 h | 24 h |
| 60 | 15.6 | 3 | 6 | 11 | 18 |
| 60 | 31.2 | 3 | 8 | 16 | 22 |
| 60 | 62.5 | 6 | 15 | 23 | 28 |
| 60 | 125 | 8 | 20 | 28 | 34 |
| 60 | 250 | 14 | 28 | 40 | 47 |
| 60 | 500 | 16 | 33 | 44 | 53 |
| 60 | 1000 | 18 | 36 | 50 | 55 |
| 60 | 2000 | 19 | 39 | 52 | 56 |

Table 2 The effect of dicamba doses on the number of deaths

| Number of individuals | Concentration (µM) | Mortality (number of dead individuals) | | | |
|---|---|---|---|---|---|
| | | 6 h | 12 h | 18 h | 24 h |
| 60 | 15.6 | 1 | 3 | 3 | 4 |
| 60 | 31.2 | 3 | 3 | 5 | 7 |
| 60 | 62.5 | 4 | 8 | 9 | 12 |
| 60 | 125 | 7 | 11 | 13 | 17 |
| 60 | 250 | 10 | 17 | 20 | 26 |
| 60 | 500 | 11 | 19 | 24 | 32 |
| 60 | 1000 | 13 | 30 | 33 | 39 |
| 60 | 2000 | 17 | 31 | 35 | 41 |

Similarly, C. elegans individuals were exposed to 8 different doses of dicamba (15.6- 31.2- 62.5- 125- 250- 500- 1000 and 2000 µM). After the lowest dose exposure of 6, 12, 18 and 24 hours, 1, 3, 3 and 4 of the C. elegans individuals died, respectively. After 6, 12, 18 and 24 hours of exposure at the highest dose, 17, 31, 35 and 41 C. elegans individuals died, respectively (Table 2). There were differences between doses in the anova and chi-square tests performed with survival numbers ($p<0.05$). In addition, paraquat and dicamba survival function graphs were created using the Kaplan-Meier method to show the differences between dose groups (Figure 1 and 2).

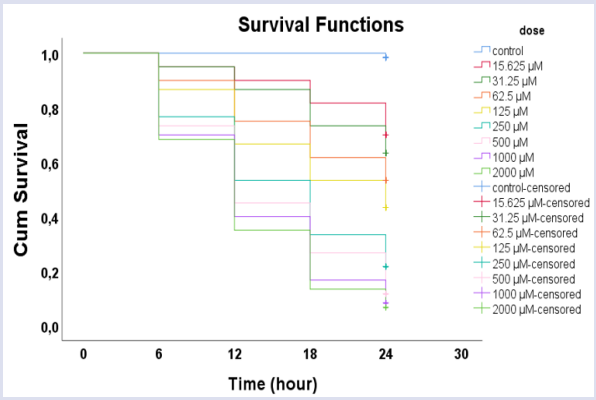



Figure 1 Paraquat survival function graph prepared with Kaplan-Meier analysis

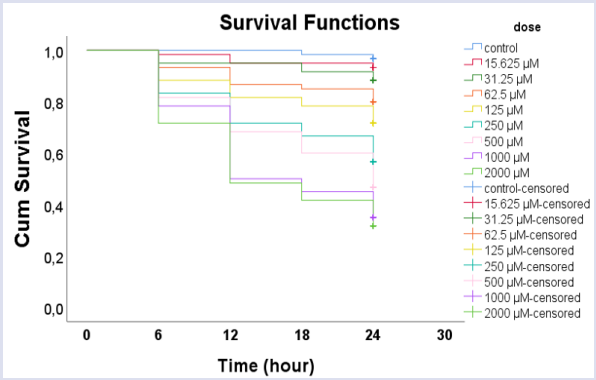
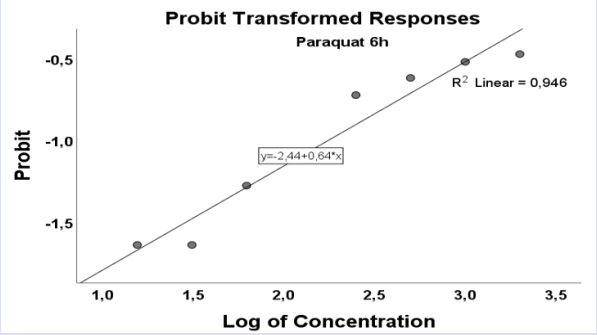
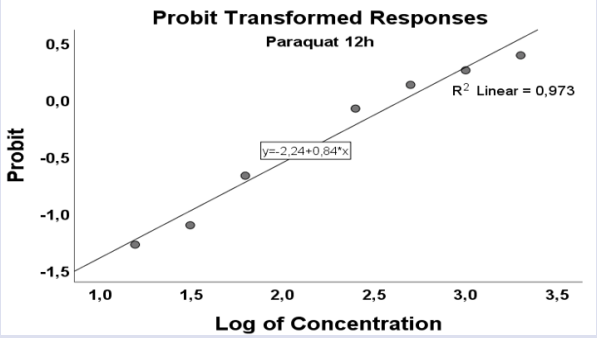


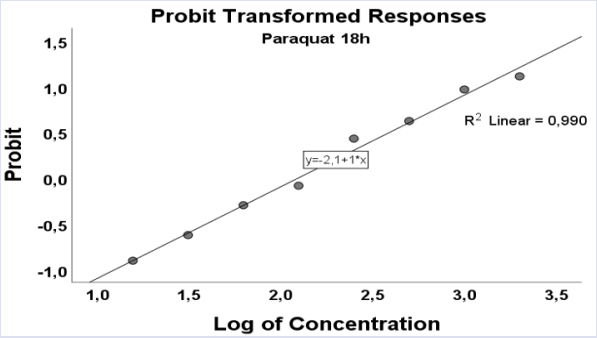

Figure 2 Dicamba survival function graph prepared with Kaplan-Meier analysis

### *C. elegans $LC_{50}$ Determination with Different Paraquat Doses*

*C. elegans* individuals were exposed to 8 different doses of paraquat (15.6- 31.2- 62.5- 125- 250- 500- 1000 and 2000 µM) for 6-12-18 and 24 hours. After exposure, *C. elegans* $LC_{50}$ values were determined by the probit test. Analysis results showed paraquat $LC_{50}$ 6h= 7412 µM, $LC_{50\ 12h}$= 459 µM, $LC_{50\ 18h}$= 123 µM, $LC_{50\ 24h}$= 61 µM (Figure 3).



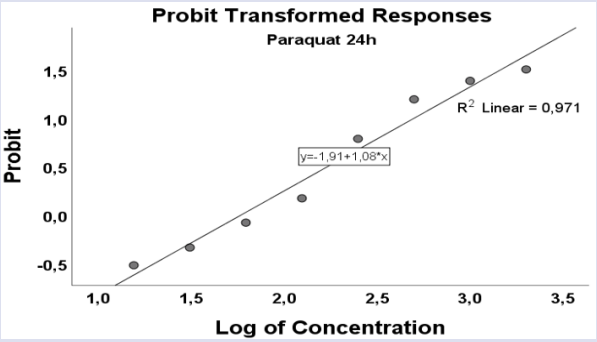
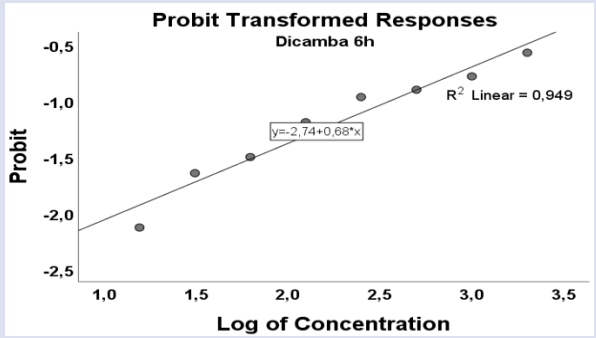
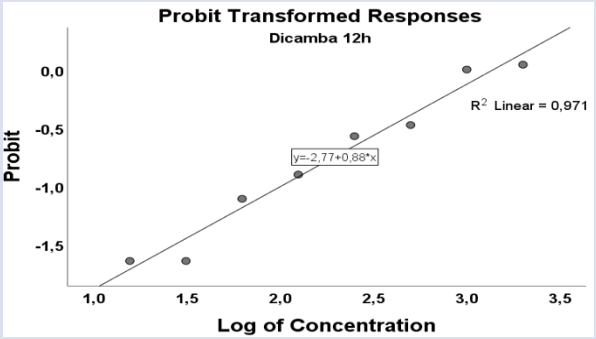
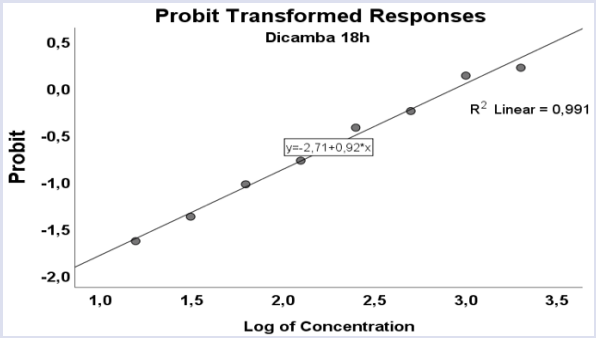




Figure 3 Paraquat dose applications probit values (6h,12h,18h and 24h)



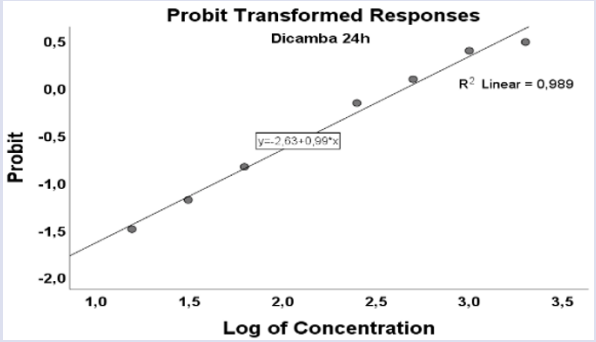




Figure 4 Dicamba dose applications probit values (6h,12h,18h and 24h)

### *C. elegans $LC_{50}$ Determination with Different Dicamba Doses*

C. elegans individuals were exposed to 8 different doses (15.6- 31.2- 62.5- 125- 250- 500- 1000 and 2000 µM) of dicamba for 6-12-18 and 24 hours. After exposure, C. elegans LC50 values were determined by the probit test. Analysis results showed dicamba LC50 6h= 14610 µM, LC50 12h= 1404 µM, LC50 18h= 906 µM, LC50 24h= 463 µM (Figure 4).

## Discussion

In our study, the number of live and dead worms was counted by visual inspection with the aid of a camera microscope [32]. At the highest dose of paraquat (2000 µM) at the end of 6,12,18,24 hours, 31.6%, 65%, 86.6% and 93.3% mortality rates were observed, respectively. In the highest (2000 µM) dose application of dicamba, 68.3%, 58.3%, 51.6% and 28.3% mortality rates were observed after 6,12,18,24 hours, respectively. When

these mortality rates are evaluated, it is seen that paraquat and dicamba have an intense toxic effect on C. elegans individuals. The fact that these toxic chemicals affect C. elegans individuals so much after 24 hours has shown that young animals are more sensitive to toxic substances than adult forms [33].

*C. elegans* is a popular living model for examining the toxicological relevance of chemical-induced toxicity at the molecular level [34,35]. In this study, $LC_{50}$ (50% lethal concentration) values of paraquat and dicamba were determined for *C. elegans*. Paraquat $LC_{50\ 6h}$= 7412 µM, $LC_{50\ 12h}$= 459 µM, $LC_{50\ 18h}$= 123 µM, $LC_{50\ 24h}$= 61 µM (Figure 3). Similarly, dicamba $LC_{50\ 6h}$= 14610 µM, $LC_{50\ 12h}$= 1404 µM, $LC_{50\ 18h}$= 906 µM, $LC_{50\ 24h}$= 463 µM (Figure 4). These determined $LC_{50}$ values showed that even a short time (6h,12h,18h,24h) exposure of *C. elegans* directly to paraquat and dicamba can cause 50% of these organisms to die.

Paraquat is a known mitochondrial toxicant. It is important to know the effects caused by mitochondrial toxicants in order to interpret lifespan analyses. In a study, using the *C. elegans* model, it was stated that paraquat affects reproduction, longevity, gene expression and mitochondrial physiology. Moreover, paraquat has been shown to induce mitochondrial unfolded protein response (mtUPR), increase mitochondrial superoxide dismutase expression, and decrease mitochondrial membrane potential (Δψm). In particular, chronic exposure to paraquata (0.035 mM) has been reported to cause gradual decline in fertility, resulting in complete loss of fertile embryo production by the third generation. Similarly, it is known that paraquat increases oxidative stress by generating reactive oxygen species (ROS), and this increased oxidative stress is one of the main causes of aging and plays a role in the pathogenesis of many diseases. In a study, it was shown that 200 mM paraquat in the NGM medium killed approximately half (LC50) of *C. elegans* individuals in the L2 and L4 form in 6 hours, and all of them in 15 hours [36]. In our study, however, paraquat was not applied in the NGM medium, and *C. elegans* individuals were directly exposed to paraquat. Therefore, the toxic effect of the applied paraquat increased and $LC_{50\ 6h}$= 7412 µM, $LC_{50\ 12h}$= 459 µM, $LC_{50\ 18h}$= 123 µM, $LC_{50\ 24h}$= 61 µM.

Although there is no study on dicamba on *C. elegans* individuals, there are some studies on the toxic effect of dicamba on some living species. In one study, the acute lethal effects of dicamba on Rhinella arenarum larvae were determined and demonstrated to induce primary DNA breaks in amphibians. In addition, dicamba was found as $LC_{50\ 24h}$ = 742 mg /L [37]. Similarly, zebrafish embryos were exposed to dicamba for 120 hours and the dicamba was found as $LC_{50\ 120h}$ = 56 mg /L [38]. When the studies on Paraquat and dicamba and our study results are evaluated together, it has been seen that LC50 values are generally directly affected by the exposure method and exposure time.

## Conclusion

As with any model system, the *C. elegans* LC50 results determined in the paraquat and dicamba toxicity screening were considered useful in predicting toxic responses in other organisms. In particular, considering the similarity of biological processes of *C. elegans* with human processes, the LC50 values of paraquat and dicamba found in the study will provide information for human toxicology studies. Similarly, the results of the study provided additional information for assessing correlations between toxicity responses in *C. elegans* and mammals, with up to 69% concordance between mammals and *C. elegans*.

## Conflicts of Interest

The authors stated that did not have conflict of interests.

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