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Araştırma Makalesi

Cytotoxic Effects of Clethodim on Different Liver Cell Lines[¥]

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ABSTRACT

Clethodim, a herbicide group pesticide, is classified as a toxicity class II agent. Up to now, the developmental toxicity, immunotoxicity, neurotoxicity, and reproductive toxicity of this herbicide have been demonstrated in zebrafish and rats. However, its hepatotoxic effects have not been reported yet in vitro. Therefore, this study was conducted for the first time to determine the effect of clethodim on human and mouse liver cell lines. We treated THLE-2 and B129 cells with a wide range of clethodim for 96 h. We evaluated its hepatotoxic effects using MTT and SRB analyses after treatment. Both assays demonstrated a striking dose-dependent decrease in cellular proliferation. In particular, a 1000 µg/ml dose of clethodim exposure stopped the growth of B129 and THLE-2 cells by 90% and 75%, respectively. Furthermore, the high doses of this herbicide significantly reduced both cell number and volume. IC_{50} doses were found to be 220 and 617 µg/ml for B129 and THLE-2 cells, respectively, indicating the more profound sensitivity of the mouse liver cells to clethodim exposure than that of human liver cells. These cytotoxic impacts can be strongly related to herbicide-induced oxidative stress. In light of our results, the long and high doses of clethodim exposure have a hepatotoxic effect, and its toxic target organ is the liver. Therefore, it is urgently needed to conduct further studies on the use of clethodim-based herbicides.

Keywords: Clethodim, human liver cell, mouse liver cell, MTT, SRB assay.

Clethodimin Farklı Karaciğer Hücre Hatlarındaki kiSitotoksik Etkileri[&]

ÖZ

Herbisit grubu pestisit olan clethodim, toksisite sınıfı II ajan olarak sınıflandırılır. Şu ana kadar,bu herbisitin gelişim toksisitesi, immünotoksisitesi, nörotoksisitesi ve üreme toksisitesi zebra balığı ve sıçanlarda gösterilmiştir.Ancak hepatoksik etkilerine yönelik invitro düzeyde henüz bir çalışma bulunmamaktadır. Bu yüzden söz konusu çalışma ilk kez clethodimin karaciğer hücre hatları üzerindeki toksik etkilerini belirlemek için yapılmıştır. THLE-2 ve B129 hücreleri, 96 saat boyunca geniş bir aralıktaki clethodim ile muamele edilmiştir. Daha sonra hepatoksik etkileri MTT ve SRB testleri ile belirlenmiştir. Her iki analiz de hücresel proliferasyonun clethodim dozuna bağlı olarak çarpıcı biçimde azaldığını ortaya koymustur. Özellikle 1000 µg/ml doz clethodim uygulamasının B129 ve THLE-2 hücrelerinde hücrel poliferasyonunu %90 ve %75 oranında baskıladığı görülmüştür Ayrıca herbisitin yüksek dozları ile hem hücre sayısında hem de hacminde önemli bir azalma elde edilmiştir. IC50 dozları B129 ve THLE-2 hücreleri için sırasıyla 220 ve 617 µg/ml olarak bulunmuştur, bu da fare karaciğer hücrelerinin clethodim maruziyetine karşı insan karaciğer hücrelerinden daha hassas olduğunu göstermektedir. Bu sitotoksik etkiler, herbisitin neden olduğu oksidatif stresle güçlü bir şekilde ilişkili olabilir. Sonuçlarımız ışığında, clethodime uzun ve yüksek dozda maruz kalmanın hepatoksik etkiye sahip olduğu ve toksik hedef organının karaciğer olduğu görülmüştür. Bu nedenle, clethodim gibi herbisit kullanımı hakkında acil olarak daha fazla çalışmaya ihtiyaç duyulmaktadır.

Anahtar kelimeler: Clethodim, insan karaciğer hücresi, fare karaciğer hücresi, MTT, SRB testleri.

INTRODUCTION

Pesticides are generally used to elevate agricultural yields by killing and inhibiting the growth of harmful organisms (Laetz et al., 2009). They are also employed in non-agricultural contexts, such as forests, alongside roads and railways, and in areas of cities. With the increased human population and crop demand, the use of natural and synthetic pesticides and their applications has profoundly increased in recent decades (Kishor et al., 2020). As a result, the exposure risk of living organisms to these substances has greatly increased. Studies have demonstrated the harmful effects of pesticide exposure on both the environment and living organisms. Pollutants, including pesticides, are known to spread quickly in the environment and human organs, leading to long-term health issues shortly after their introduction (Gavrilescu et al., 2015). Because of their significant metabolic activities, the liver is the main target organ for pesticide damage (Çakır and Erden, 2019). Hence, it is a global priority to protect human health from pesticides, pesticide-contaminated drinking water, and harmful air pollutants (Damalas and Koutroubas, 2016). On the contrary, their action mechanisms, especially their cytotoxic effects, are not fully known. Among the pesticides, clethodim is a very effective one, as is the cyclohexanedione class of herbicides (Santos et al., 2023). Furthermore, it is the primary active constituent in prevalent herbicidal formulations. Therefore, it frequently targets both annual and perennial grasses by inhibiting acetyl-coenzyme A carboxylase. Substantial annual sales surpassing $100 million are also evidence of its widespread global application (Mohamed et al., 2023). The EPA classifies it as a toxicity class II agent, indicating that it is moderately toxic and moderately irritating. Due to its high solubility in water, it can easily enter the aquatic environment. Consequently, it has effects on aquatic organisms since the photodegradation products of the clethodim are stable in the aquatic environment (Hong et al., 2023). The toxicity studies of clethodim in vivo revealed that its exposure induces developmental toxicity and neurotoxicity in zebrafish embryos and larvae (Wang et al., 2019). Researchers have also reported the negative effects of this herbicide exposure on male reproductive function and early embryogenesis in Swiss albino mice due to its endocrine-disrupting function (Dcunha et al., 2023). Furthermore, clethodim can cause irritation to the eyes, skin, and central nervous system when encountered in high amounts. This can result in symptoms such as excessive salivation, reduced motor activity, a lack of coordination, unstable walking, and increased activity levels (Pang and Hu, 2020). Conversely, chronic toxicity assessments of this compound indicated anemia, increased hepatic mass, skeletal damage, and decreased fetal body weight (Wang et al., 2019). Additionally, they demonstrated the inhibition of the acetyl coenzyme A carboxylase activity in the C_2C_{12} myoblast cell line (Patil et al., 2007). Despite numerous studies on the toxic effects of clethodim, its target organ and toxicity mechanisms remain unclear. Thus, we conducted this study for the first time to reveal the cytotoxic effect of this herbicide on the mouse and human liver cell line models since the liver plays a crucial role in the metabolism and biotransformation of pesticides. Moreover, cell culture offers a valuable platform for the meticulous evaluation of pharmaceutical agents, enabling the systematic exploration of drug effects alongside its pivotal role in toxicological studies (Holen et al., 2010).

MATERIAL AND METHODS

Cell cultures and chemicals

The THLE-2 (CRL-2706) human liver cell line was gifted from Prof. Gokhan Sadi's lab at Karamanoğlu Mehmetbey University. Additionally, we obtained the B129 mouse hepatocyte cell line from Cell Biologics (USA). Unless explicitly indicated otherwise, Sigma-Aldrich, Inc. (St. Louis, MO, USA) provided all analytically pure chemicals used in the research.

The growth of cell cultures and their exposure to clethodim

We cultured the THLE-2 and B129 cells in DMEM high glucose supplemented with 100 IU/ml penicillin, 100 g/ml streptomycin, and 10% fetal bovine serum. Both cell lines were grown in the incubators at 37°C with 95% air and 5% CO_2. We trypsinized them after they reached 75–80% confluency. We then cultured them in 96-well plates at a density of $1x10^4$ per 100 µl well for 24 h before clethodim treatment.

Clethodim Exposure

We made a stock solution of clethodim (LGC, TRC-C573250) by mixing 100 mg of the herbicide with 100 µl of dimethyl sulfoxide (DMSO). The liver cell lines were grown in a 96-well plate for 24 hours and then

exposed to a range of clethodim concentrations (125–1000 µg/ml) for 96 hours. We used a 0.1% DMSO-exposed as a negative control. We used 500 µM H_2O_2 treatment as a positive control because it rapidly causes apoptosis and necrosis in cells. Its effects on cells are also well-characterized and relatively cheap with respect to the chematherapeutic drugs (Saito et. al., 2006).

Cell viability assays

We first determined the cell viability using the MTT assay based on a previous study (Nordin et al., 2019). After 96 h of clethodim incubation, 20 µl of 5 mg/ml MTT in 100 µL of culture medium was added to each well. The plates were then put in an incubator with 5% CO_2 at 37°C. After 4 h of incubation, the media was discarded, and 150 µl of DMSO was added to dissolve the formazan crystals. We used an ELISA reader (Epoch microplate reader, BioTek, USA) to measure the absorbance of the soluble formazan product from living cells at 570 nm. We also used an SRB (Sulforhodamine B) assay to verify the MTT assay. We first treated the cells with clethodim for 96 h, mimicking the MTT assay. Then we added 10% trichloroacetic acid (TCA) to fix the proteins in the cell. We kept the plate at +4 ºC for 1 hour, then washed it with distilled water and dried it at room temperature for 20 minutes. Then we stained the cells with a 0.4% SRB solution for 30 minutes at room temperature and washed the wells with 1% acetic acid four times to remove unbound SRB. Later, we added Tris-base (pH: 10) into each well to dissolve the SRB dye. We measured the absorbance of SRB at 574 nm using the same microplate reader. We considered the viability rate of control cells to be 100% in both assays and calculated the viability using the formula below.

% Viable cells = (Absorbance of cell treated with clethodim) - (Absorbance of empty well) / (Absorbance of control cell) - (Absorbance of empty well) x 100

Statistical data analysis

To compare the statistical significance in cell viability between the control and clethodim-treated groups, we performed the cytotoxicity experiments in two independent experiments. Data are presented as mean ± S.E.M. We used the one-way analysis of variance (ANOVA) followed by a Turkey's test using GraphPad Prism statistical software version 6.0 (Graph Pad Prism, San Diego, CA, USA). We accepted $P < 0.05$ to indicate significant differences between groups. We also calculated the IC_{50} values using the same statistical software.

RESULT

Cell culture lines have been widely used in the toxic evaluation of any chemical, including pesticides, because they are easy to obtain and produce reproducible results (Holen et al., 2010). Hence, we used the mouse and human hepatic cell lines.

In cell culture studies, the MTT assay is a well-established method to determine cell viability, proliferation rate, and cytotoxicity. This method is based on the ability of metabolically active cells to transform a water-soluble dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into an insoluble purple formazan (Ghasemi et al., 2021). In this assay, DMSO is used to solve formazan crystals (Bahuguna et al., 2017). The cellular viability is described by reading the absorbance value of the purple color intensity using an ELISA reader. We first used this method to depict the effect of clethodim on the viability of the liver cells. We have first tried the cells with the lower dose of clethodim (<100 µg/ml). However, we did not observe any reduction in cell proliferation with these doses (data not shown). Therefore, we treated the studied cells with a range of concentrations (125, 200, 250, 400, 500, 800, and 1000 g/ml) for 96 h. Figure 1A displays the cell viability results for the B129 cells in the control and clethodim-exposed groups, while Figure 1B displays the same results for the THLE-2 cells. Both figures clearly show a significant decline in both cells' viability, which is concentration-dependent. At the dose of 1000 µg/ml, the lowest cell viability was obtained for B129 (7%, $p<0.001$) and THLE-2 (19%, $p<0.001$) cells. Based on the MTT results, we can assume that B129 cells were profoundly affected by the clethodim exposure with respect to the THLE-2 cells.

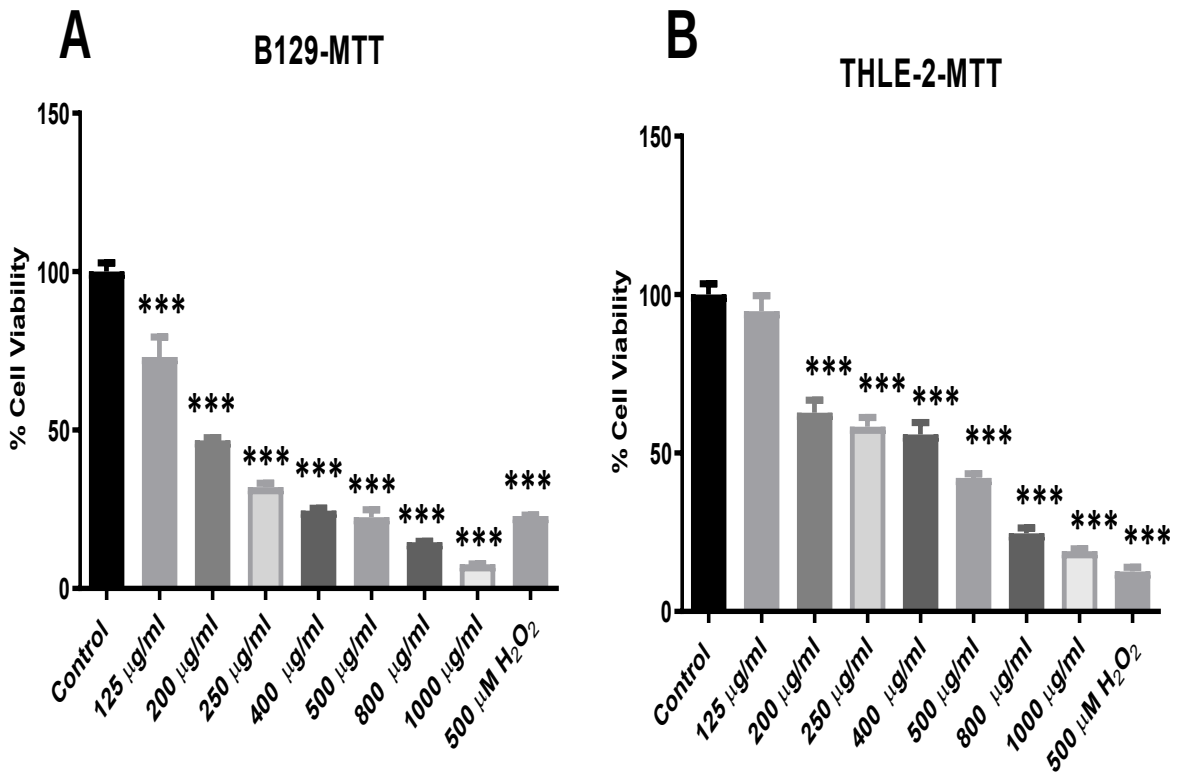

Figure 1. The cell viability of B129 (A) and THLE-2 cells for the control and clethodim treatment (125–1000 µg/ml) based on the MTT assay. Each value represents the mean ± S.E.M. of two independent experiments. "*** p<0.001" denotes significant differences with respect to the control group.

To validate MTT cytotoxicity results, we utilized the SRB (Sulforhodamine B) assay. This assay was developed by Skehan et al. (1990). That has been widely employed to conduct cost-effective screening assays in cell-based research for investigating cytotoxicity (Vichai and Kirtikara, 2006). This approach utilizes the characteristic of SRB, which forms a stoichiometric bond with proteins under mildly acidic conditions and can subsequently be removed using basic conditions. Consequently, the quantity of dye attached to proteins can serve as an indicator of cell mass, enabling the extrapolation of cell proliferation measurements. Concerning the MTT, the SRB assay exhibited superior linearity with cell number and increased sensitivity, and its staining was not influenced by the type of cell line (Keepers et al., 1991). Therefore, it has gained popularity among cell viability assays for two decades. Because of these advantages, we performed a SRB assay to validate the hepatotoxic effects of the clethodim with high doses, like what was seen with MTT. We saw that the clethodim treatment decreased the growth of both types of liver cells in a dose-dependent way. For THLE-2 cells, SRB assay results seemed to be more accurate when compared to the MTT method. Especially at the highest dose of clethodim, approximately 29% cell viability was found in human liver cells. SRB assay results confirmed that B129 is more vulnerable to this herbicide than THLE-2 cells (Figure 2).

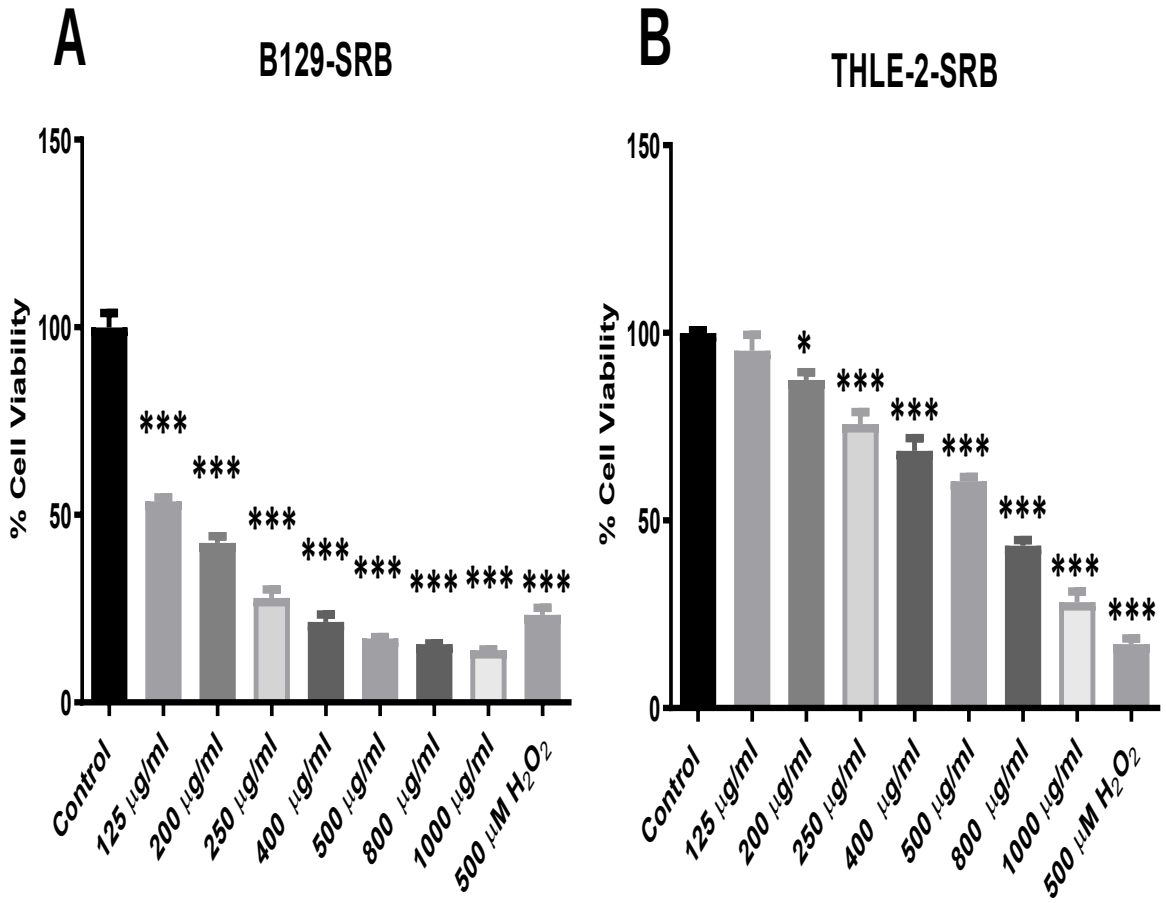

Figure 2. The cell viability of B129 (A) and THLE-2 cells for the control and clethodim treatment (125–1000 µg/ml) based on the SRB assay. Each value represents the mean ± S.E.M. of two independent experiments. "*p<0.05,*** p<0.001" denotes significant differences with respect to the control group.

To be sure about the clethodim-induced hepatotoxic effect, we collected the SRB-stained cells for the control, 125 and 800 µg/ml doses of clethodim, and 500 µM H_2O_2-treated cells. Figures 3 and 4 demonstrate the light microscopic images of B129 and THLE-2 cells, respectively. As can be clearly seen from both figures, the 800 µg/ml dose of clethodim significantly decreased cell proliferation and cellular shape in both B129 and THLE-2 cells. These alterations were found to be striking for B129 cells. To evaluate the difference in the cytotoxic effects of clethodim between human liver and mouse liver cells, we calculated the IC_{50} doses of this herbicide. The IC_{50} doses were found to be 220 and 617 µg/ml for B129 and THLE-2 cells, respectively. This finding also supports the idea that mouse liver cells are more sensitive to clethodim exposure than human liver cells.

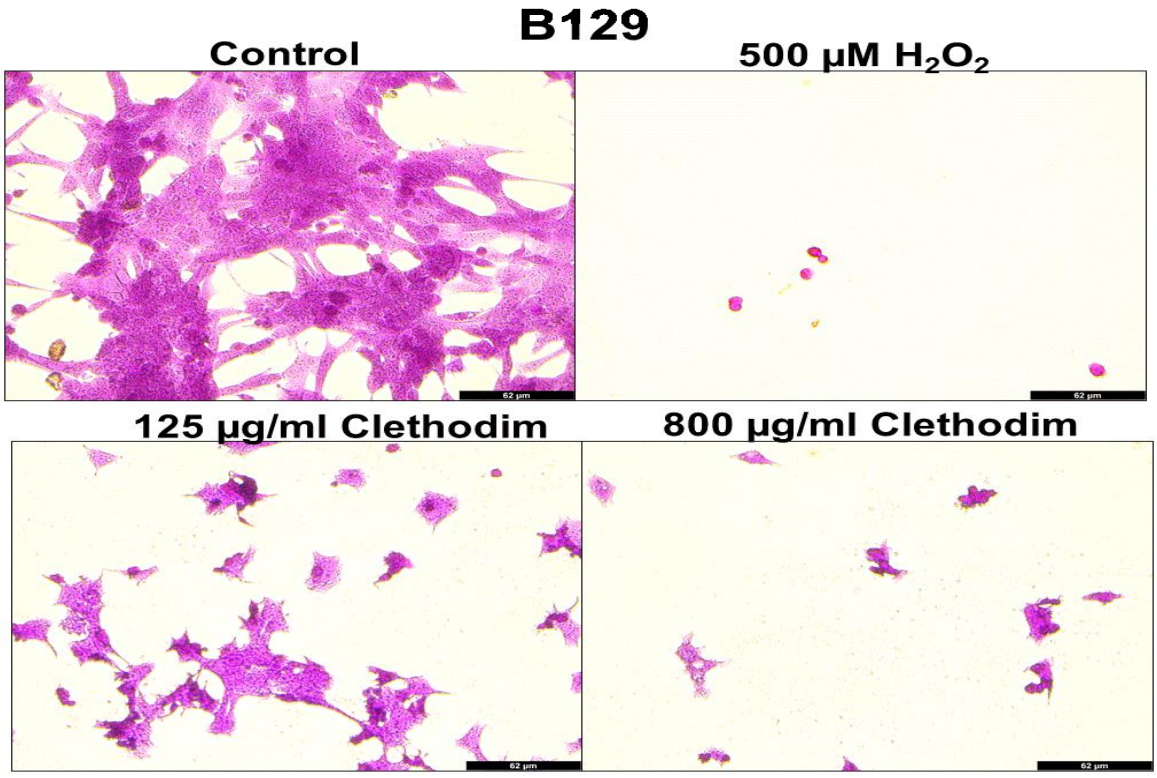

Figure 3. The micrographs of the control and clethodim-treated B129 cells obtained from the SRB assay. Cell morphology was captured by light microscopy (10X objective).

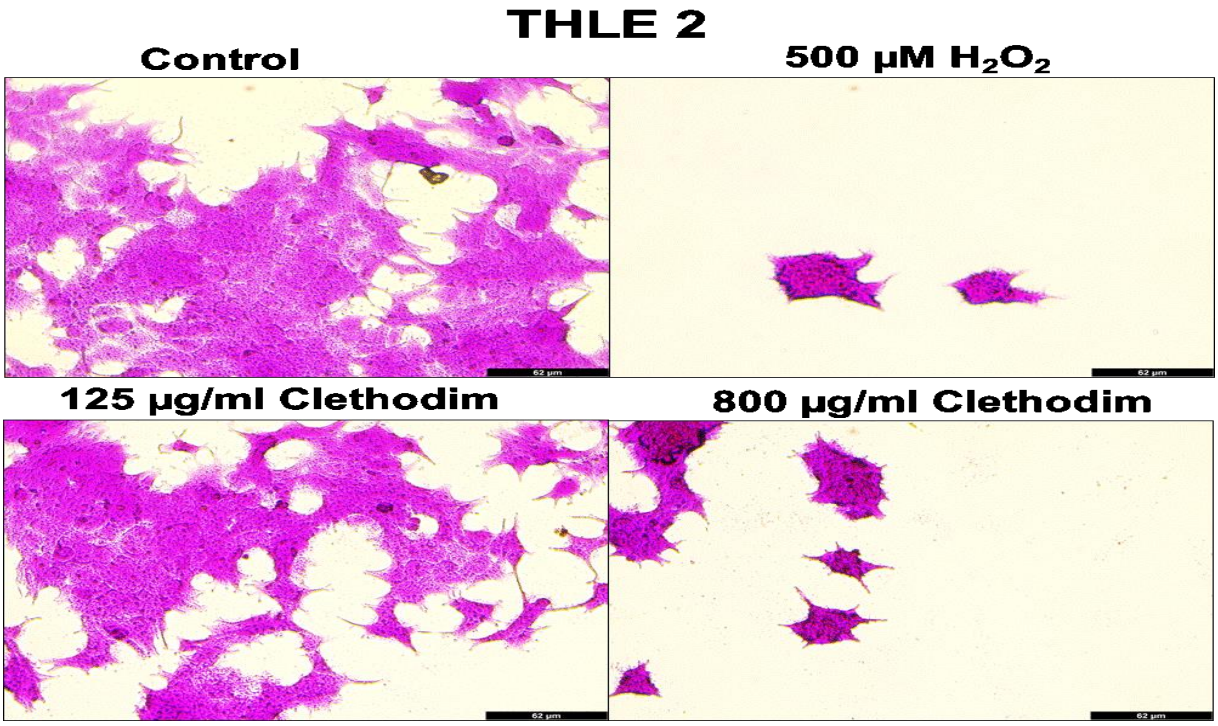

Figure 4. The micrographs of the control and clethodim-treated THLE-2 cells obtained from the SRB assay. Cell morphology was captured by light microscopy (10X objective).

DISCUSSION

Clethodim is a commonly used herbicide in agricultural and non-agricultural areas due to its high efficacy and safety with low mammalian toxicity (Mohamed et al., 2023b). To date, researchers have conducted a few studies on mice and zebrafish animal models to investigate the toxic effects of this compound (Dcunha et al., 2023; Xiong et al., 2019). There is no information about the target organ or its toxic mechanisms. Moreover, there is no study about the hepatotoxic effects of the clethodim, especially at the in vitro cell culture level. For that reason, we carried out this study to determine the cytotoxic effects of clethodim on two different liver cell lines: human and mouse. MTT and SRB cell viability findings elucidated that the high doses of the clethodim have hepatotoxic effects. Similar to these effects, studies on the developmental, neurotoxic, and immunotoxic effects of clethodim on zebrafish embryos and mice have been published (Dcunha et al., 2023; Wang et al., 2019; Xiong et al., 2019). Supporting the study, the hepatotoxic effect of this herbicide was also demonstrated by Alaa M. H. Khozimy (2021). In that study, clethodim (163 and 326 mg/kg body weight) herbicides were orally administered to male albino rats for 28 days. The study found that the administration of clethodim significantly increased the levels of liver enzymes (ALT and AST) and the weight of liver organs. In the same study, to detect the clethodim associated with oxidative stress, they measured hematological parameters such as red blood cells (RBC), white blood cells (WBC), and hemoglobin. A sub-lethal dose of clethodim treatment induced a decrease in RBC and hemoglobin and a large rise in WBC. Based on these results, the cytotoxic effect of the clethodim may be associated with the herbicide's elevated ROS levels in liver cells since enhanced ROS levels are known to degrade cellular macromolecules such as lipids, proteins, DNA, etc. (Andrés Juan et al., 2021). This disruption may lead to the loss of the cellular main functions, so they enter the apoptotic/necrotic phase, and so on. Confirming this hypothesis, the hepatotoxic molecular mechanism of 2,4-dichlorophenoxyacetic acid (2,4-D) was reported in a review performed by Martins et al. (2024). In that study concluded that 2,4-D damages several hepatic biochemical markers, particularly elements of the antioxidant system, and that oxidative damage likely significantly contributes to the formation of 2,4-D-induced liver damage. Therefore, we can infer that clethodim-associated hepatotoxicity may be associated with these mechanisms (Brêda-Alves et al., 2020).

CONCLUSION

This study shows that high doses of clethodim are toxic to the liver, as shown by the MTT and SRB cell viability assays. Our results underscore the urgent need for immediate care when using clethodim-based herbicides. Furthermore, it is essential for additional studies to explore the health consequences of long-term exposure, specifically at lower levels.

¥: This study was produced from the master's thesis of the first author.

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Disclosure statement

The authors declare that they have no conflicts of interest.

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