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Effects of Imazethapyr-Based Herbicide Formulation in the Zebrafish (*Danio rerio*) Hepatocyte Cell Line (ZF-L): Cytotoxicity and Oxidative Stress

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Abstract

Seizures of agrochemical formulations have increased in Brazil and Rio Grande do Sul is among the Brazilian states with the highest number of seizures of these products obtained illicitly. The use of illicit formulations can cause significant harm to agricultural production, the environment, and non-target species. This study evaluated the cytotoxicity and oxidative stress of a seized formulation containing the herbicide imazethapyr (IMZT). Characterization of the herbicide included gas chromatography-mass spectrometry (GC-MS) and thermal analyses (thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)). Hemolytic and cytotoxicity assays in ZF-L hepatic cells showed IC_{50} values of 12.75 μg/mL, 3.01 μg/mL, 2.67 μg/mL, and 1.61 μg/mL for erythrocytes, [3(4,5-dimethyl)-2 bromide-5 diphenyl tetrazolium] (MTT), neutral red (NR), and lactate dehydrogenase

(LDH) assays, respectively. The median IC_{50} of 2.84 μg/mL was used in oxidative stress assays, revealing increased reactive oxygen species (ROS) production, reduced total sulfhydryl content, and decreased superoxide dismutase (SOD) and catalase (CAT) activity. This study is the first to report *in vitro* oxidative stress induced by IMZT in the ZF-L cell line, emphasizing the importance of *in vitro* assays for assessing the toxic effects of seized agrochemicals on human health and the environment.

Keywords

Agrochemical, Imidazolinone, Zebrafish, Cell Viability, Oxidative Damage

1. Introduction

Agrochemicals are substances toxic to most organisms, whether terrestrial or aquatic and are designed to combat or control pests, aiming to improve agricultural production [1]. However, the intensive and indiscriminate use of agrochemicals contributes to contamination not only of food but also of various environmental compartments such as soil, water, and air [2]. Herbicides are a class of agrochemicals used for the control and elimination of unwanted plants, playing a crucial role in large-scale agricultural production [2].

Herbicide formulations containing the active ingredient IMZT [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5-dihydroimidazol-1H-2-yl) nicotinic acid] are widely used in the agricultural sector in various countries [3]-[7]. This herbicide is recommended for pre- or post-emergence control of weed plants in crops such as rice, soybeans, among others [3] [8] [9]. Herbicides belonging to the imidazolinones class, such as IMZT, are designed to target specific plant mechanisms and are considered to have low acute toxicity [10]. However, several studies have shown adverse effects on non-target organisms [3] [5] [6] [8]-[11].

Brazil is one of the countries that consume the most agrochemicals in the world and Rio Grande do Sul is among the Brazilian states with the highest number of seizures of these products obtained illicitly, via smuggling [12] [13]. According to Moraes (2022) [14], between 2007 and 2018, Brazilian federal agencies seized approximately 1.3 thousand shipments of contraband agrochemicals.

Forensic experts primarily use chemical identification procedures to analyze seized agrochemicals [15] [16]. Additionally, the toxicity of agrochemicals is extensively studied both *in vitro* and *in vivo* [3] [17]. However, there are no reports on these evaluations for seized formulations.

The use of *in vitro* models, including fish liver cell cultures, for cytotoxicity testing has increased in recent years, given that the liver is the primary target organ for most toxins [18] [19]. In this context, the ZF-L cell line, derived from the normal liver tissue of zebrafish (*Danio rerio*), is recommended for *in vitro* studies of metabolism and, consequently, xenobiotic metabolite formation [17]

[20]-[23]. Additionally, several studies have demonstrated that many physiological mechanisms between zebrafish and mammals can be similar [24]-[28].

Therefore, in this study, we evaluated the *in vitro* cytotoxicity and oxidative stress caused by a formulation containing the seized herbicide IMZT in the Southern region of Brazil, using the ZF-L cell line. To date, this is the first study conducted with this herbicide type using this cell line for the toxicological analysis of seized herbicides.

2. Materials and Methods

2.1. Chemical Reagents

Leibovitz's L-15 and Ham's F-12 media were obtained from Vitrocell Embriolife (Campinas, SP, Brazil). Dulbecco's Modified Eagle's Medium (DMEM) with high glucose content was obtained from Inlab Diagnóstica (Diadema, SP, Brazil). Defibrinated sheep blood was purchased from Laborclin (Pinhais, PR, Brazil). Trypsin/EDTA solution, Fetal Bovine Serum (FBS), and 3(4,5-dimethyl)-2 bromide-5 diphenyl tetrazolium (MTT) were obtained from Gibco (Gibco, Carlsbad, CA, United States). All other reagents and solvents used were of analytical or pharmaceutical grade. The IMZT sample was kindly provided by the Brazilian Federal Police (PF), after a seizure at the Brazil/Uruguay border, in the city of Jaguarão (Brazil), according to Berneira *et al.* [15].

2.2. Agrochemical Extraction and Identification

90 mg of the agrochemical formulation was extracted with 25 mL of acetonitrile. The material was centrifuged for 5 min, and 1 μL of the solvent fraction was injected into a GC-MS QP2010SE (Shimadzu®) in split mode (1:25). Helium was used as the mobile phase and the Rtx-5MS was used as the capillary column. The temperature was set at 260˚C for the injector and 280˚C for the ion source. The initial temperature of the column was 200˚C for 12 min, with a 5˚C/min heat ramp until 280˚C, then holding for 5 min. MS ran in scan mode from 50 to 550 m/z [15].

2.3. Thermogravimetric Analysis (TGA)

The thermal stability of the IMZT herbicide formulation was determined using the TGA Q5000 equipment (TA Instruments Inc., USA). For this analysis, the heating rate was 10˚C/min using an inert N_2 atmosphere (25 mL/min). The equipment was calibrated with $CaC_2O_4{\cdot}H_2O$ (99.9%). The mass of the sample was 4.473 mg. Data were processed using TA Universal Analysis 2000 Software, version 4.5 (TA Instruments Inc., USA).

2.4. Differential Scanning Calorimetry (DSC)

Modulated Temperature Differential Scanning Calorimetry (MT-DSC) was performed on DSC Q2000 equipment (TA Instruments, USA) with MTDSC option, with RCS cooling and N_2 as purge gas (50 mL/min), which was used to deter-

mine DSC. The heating rate was 5˚C/min. The instrument was initially calibrated in the DSC standard form, using indium (99.99%). The sample mass (11.384 mg) was weighed with an accuracy of (±0.001 mg). Data were processed using TA Universal Analysis 2000 Software, version 4.5 (TA Instruments Inc., USA).

2.5. *In Vitro* Cytotoxicity Assays

The cytotoxicity of the herbicide was initially evaluated in sheep erythrocytes and then using ZF-L cells.

2.5.1. Hemolytic Activity Assay

The hemolytic activity (HA) induced by IMZT was evaluated spectrophotometrically in a hemoglobin release assay, using defibrinated sheep's blood (Laborclin®), as described by Cerveira *et al.* [29]. Briefly, we used a suspension of 4% (v/v) of red blood cells in 0.9% NaCl. This solution was then incubated with IMZT at dilutions of 4.82, 9.64, 19.29, 38.58, 77.16, 154.32, and 308.64 µg/mL at 37˚C for 1 h and centrifuged for 10 min at 2500 rpm. The supernatants were transferred to a 96-well plate and the absorbance was read at 419 nm. For negative and positive control, saline solution, and Triton-X 100 (0.1%) were used, respectively. The percentage of hemolysis was calculated as (AT − AC)/(AX − AC) × 100, where AT is the absorbance of the treated supernatant, AC is the absorbance of the negative control and AX is the absorbance of the positive control.

2.5.2. ZF-L Cell Line Culture

The Zebrafish-Liver (ZF-L) cell line was acquired from the Rio de Janeiro Cell Bank (BCRJ – Brazil). Cells were grown in culture flasks and maintained in 50% L-15 (Vitrocell®, Brazil), 35% DMEM high glucose (INLAB®, Brazil), 15% Ham's F-12 (Vitrocell®, Brazil), supplemented with 0.15 g/L $NaHCO_3$, 15 mM HEPES, 0.01 mg/mL insulin, 7% (v/v) heat-inactivated FBS (Gibco®, Brazil), 50 µg/mL enrofloxacin and 2.5 µg/mL amphotericin B. The cells were cultured and maintained in a dry oven at 28˚C.

2.5.3. Cell Culture Exposure

For cytotoxicity and oxidative stress assays, ZF-L cells were seeded in 96-well plates at a density of 3×10^4 cells/well and in 6-well plates at a density of 6×10^5 cells/well, respectively. The plates were incubated for 48 hours for complete cell adhesion. The formulation containing the IMZT herbicide was diluted in a complete medium without the addition of FBS immediately before each experiment. For cytotoxicity assays, concentrations were defined based on the results determined in the IC_{50} of the HA, and they were set at 0.44, 0.88, 1.75, 3.51, 7.01, 14.03, and 28.06 µg/mL. To determine oxidative stress in ZF-L cells, concentrations of 1/2×, 1×, and 2× corresponding to the median IC_{50} of the cytotoxicity assays were used. Triton X-100 at 5% was used as the positive control (PC) for the LDH assay, and H_2O_2 at 0.5% for MTT, NR assays, and oxidative stress tests.

Cells treated only with culture medium were used as the negative control (NC) for all assays. The treated cells were incubated under the same conditions for 24 hours.

2.5.4. Mitochondrial Viability Assay

Mitochondrial viability was determined according to the method described by Mosmann [30] using MTT. This assay is based on the ability of mitochondria to reduce MTT (yellow) to blue formazan crystals. After the cell incubation period at the tested concentrations, the supernatant was removed, and the cells were washed with PBS. The MTT solution (1 mg/mL) was added 50 µL/well and incubated in an oven at 28˚C, for 3 h. The medium was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was recorded at 540 nm using a microplate reader (SpectraMax M3). The percentage of viability was calculated as AT/AC × 100; where AT and AC are the absorbances of the treated and control cells, respectively.

2.5.5. Neutral Red Dye Uptake Assay

Lysosome integrity was measured using the neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) uptake assay according to the method described by Borenfreund and Puermer [31], with modifications. This assay evaluates the ability of the lysosome of viable cells to incorporate and retain neutral red. After the cell incubation period at the tested concentrations, the cells were washed with PBS, and 200 µL/well of neutral red solution (40 µg/mL) was added and incubated in an oven at 28˚C for 3 h. After the incubation period, the cells were washed twice with PBS again to remove the dye that was not incorporated into the lysosomes. Then, we added 150 µL/well of the acid-alcohol solution (50% ethanol, 49% distilled water, and 1% glacial acetic acid). Absorbance at 540 nm was determined using a microplate reader (SpectraMax M3). The percentage of viability was calculated using the formula AT/AC × 100; where AT and AC are the absorbances of the treated and control cells, respectively.

2.5.6. Lactate Dehydrogenase Release Assay

The enzyme release assay induced by the action of IMZT on the plasma membrane of ZF-L cells was determined using the lactate dehydrogenase (LDH) assay, as described by Vaucher *et al.* [32] with modifications. After the incubation period of the cells at the tested concentrations, the supernatants were collected, and the release of LDH. This test was performed through an enzymatic kinetics assay using a commercially available LDH (UV) kit (Bioclin®– Quibasa Ltda, Belo Horizonte, MG, Brazil). Absorbance at 340 nm was determined using a Cobas MIRA® automated analyzer (Roche Diagnostics, Basel, Switzerland), following the manufacturer's instructions. The percentage of LDH release was calculated following the equation: (AT − AC)/(AX − AC) × 100, where AT is the absor-

bance of treated cells, AC is the control absorbance of untreated cells and AX is the absorbance of cells lysed with Triton X-100.

2.6. Oxidative Stress Parameters

2.6.1. Lysate Preparation

After the incubation period of the cells at concentrations of 1/2× (1.42 µg/mL), 1× (2.84 µg/mL) and 2× (5.68 µg/mL) corresponding to the median IC_{50} value, the cells were washed twice with PBS. The lysate was prepared using a mechanical scraper. We then centrifuged at 1000 rpm for 10 min at 4˚C. The pellet was discarded, and the supernatant was used to evaluate oxidative stress parameters (SOD, CAT and –SH). Protein levels were measured using bovine serum albumin as a standard, as described by Lowry *et al.* [33].

2.6.2. Determination of Reactive Oxygen Species (ROS)

Intracellular ROS generation in intact cells is based on the oxidation of dichlorohydrofluorescein 2'-7'-diacetate (DCFH-DA) to fluorescent 2',7'-dichlorofluorescein (DCF) and was determined by the DCFH assay as described by Ali *et al.* [34]. Briefly, after the incubation period of cells at concentrations of 1/2× (1.42 µg/mL), 1× (2.84 µg/mL), and 2× (5.68 µg/mL) corresponding to the median IC_{50} value, in 96-well plates at a density of 3×10^4 cells/well, cells were incubated with 1 µM DCFH-DA for 30 min. Fluorescence was measured with excitation/emission at 488/525 nm in a microplate reader (SpectraMax M3). ROS production was expressed as a percentage of control (NC).

2.6.3. Quantification of Total Sulfhydryl Content (-SH)

Total sulfhydryl content in cell lysates was determined according to Aksenov and Markesbery [35]. This test is based on the reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols. This reaction forms an oxidized disulfide generating a yellow derivative (TNB). The reaction was initiated by the addition of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). Absorbance was measured at 412 nm in a microplate reader (SpectraMax M3) and results were expressed as a percentage of control (NC).

2.6.4. Superoxide Dismutase (SOD) Activity

SOD activity in cell lysates was assessed as described by Misra and Fridovich [36]. Catalase (10 µM), glycine buffer (50 mM, pH 10.2), and adrenaline (60 mM) were added to the samples. Absorbance was measured at 480 nm in a microplate reader (SpectraMax M3) and the results were expressed as a percentage of the control (NC).

2.6.5. Catalase (CAT) Activity

CAT activity in cell lysates was quantified by the method described by Aebi [37]. This process is based on the decomposition of 30 mM hydrogen peroxide (H_2O_2) in 50 mM potassium phosphate buffer (pH 7.0). The reaction was continuously monitored at 240 nm for 180 s at 37˚C. Reading was performed on a microplate

reader (SpectraMax M3) and the results were expressed as a percentage of control (NC).

2.7. Statistical Analysis

Data analysis was performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), for the HA, MTT, NR, and LDH assays. Data were expressed as the mean ± standard deviation for triplicates using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. IC_{50} was calculated by non-linear regression analysis for HA, MTT, NR, and LDH assays. For oxidative stress parameters, data were subjected to one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons. Differences between mean values were considered significant when $p < 0.05$.

3. Results

3.1. Compound Identification

The herbicide IMZT was identified through GC-MS, and its chromatogram is shown below in Figure 1. The peak obtained for IMZT in the chromatogram was confirmed by its mass spectra (data not shown). All the other peaks are components of the formulation.

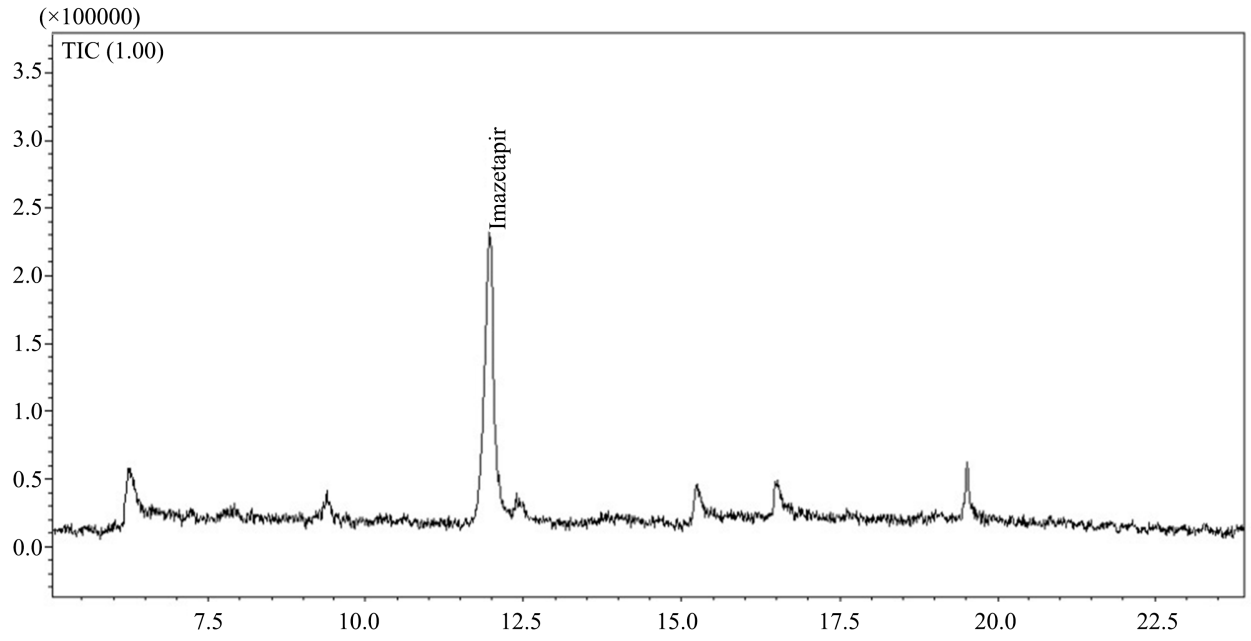


Figure 1. Representative chromatogram of IMZT.

3.2. Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC)

The results of thermogravimetric analysis (TGA) and the derivative curve (DTG), as well as the differential scanning calorimetry (DSC) of IMZT, are presented in Figure 2.

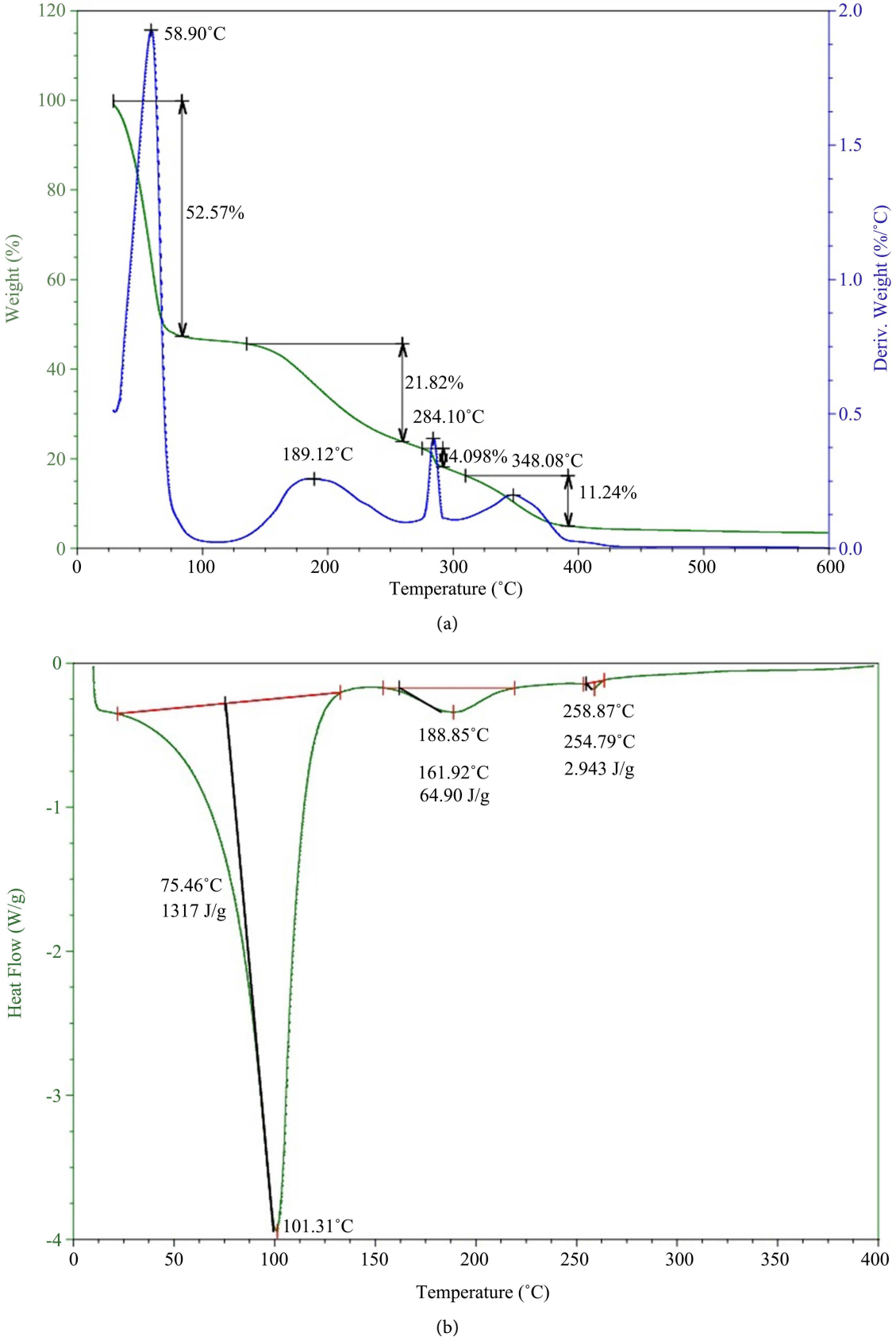


Figure 2. Thermal response curves for the IMZT herbicide formulation. (a) TGA and DTG curve; (b) DSC curve.

In the TGA and DTG analysis, the peaks indicate the thermal events occurring in the TGA curve, confirmed by the DTG curve of mass loss concerning temperature. As observed in **Figure 2(a)**, this herbicide exhibited four mass loss events. The first event involved a mass loss of 52.57% at a midpoint temperature of 58.90˚C. The second event showed a loss of 21.82% at a midpoint of 189.12˚C. The third event had a decrease of approximately 4% at a midpoint of 284.10˚C. The fourth event involved a loss of 11.24% at a midpoint temperature of 348.08˚C. Finally, after 400˚C, no appreciable mass loss was observed. These mass losses could be attributed to thermal decomposition reactions such as decarboxylation and the bond breakage between the two heterocycles in the IMZT molecule.

The result of the DSC of herbicide is presented in **Figure 2(b)**. Physical-chemical transitions of the sample were observed, as indicated by energy variations concerning temperature. Three events occurred: the first, at a midpoint temperature of 101.31˚C with an enthalpy variation of 1317 J/g at 75.46˚C; the second at a midpoint temperature of 188.85˚C, with an enthalpy variation of 64.90 J/g at 161.92˚C; and finally, an enthalpy variation of 2.943 J/g at 254.79˚C occurred at a peak temperature of 258.87˚C (**Figure 2(b)**).

3.3. Cytotoxicity Assays

3.3.1. Hemolytic Activity

The effect of the IMZT herbicide formulation on hemolytic activity is illustrated in **Figure 3**. The activity was determined as the percentage of lysis of defibrinated sheep blood erythrocytes. According to the data obtained in this study, it was observed that IMZT demonstrated the ability to induce hemolysis above 50% at concentrations of 19.29 to 308.64 μg/mL (70.44%, 99.23%, 99.74%, 100.18%, and 100.51%, respectively). At concentrations of 38.58 and 308.64 μg/mL, hemolysis similar to PC (100%) was observed. The IC_{50} in this assay was 12.75 μg/mL.

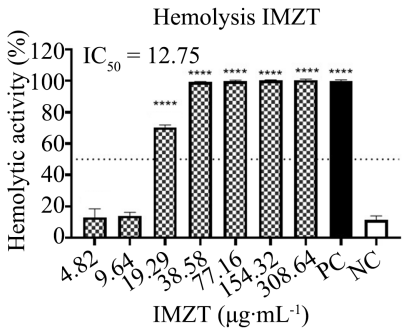


Figure 3. Hemolytic activity of IMZT in animal erythrocytes. Data is expressed as mean ± SD, whereas the mean of positive control was used as 100%. Triton X-100 and saline were used as positive and negative controls, respectively. Data were analyzed using a one-way ANOVA followed by Dunnett's post-hoc test to determine the significance level where ****$p < 0.0001$, in comparison to the positive control (PC). The inhibitory concentration value (IC_{50}) is also presented and was calculated using the non-linear regression method, based on concentration and percentage of activity.

3.3.2. MTT Assay

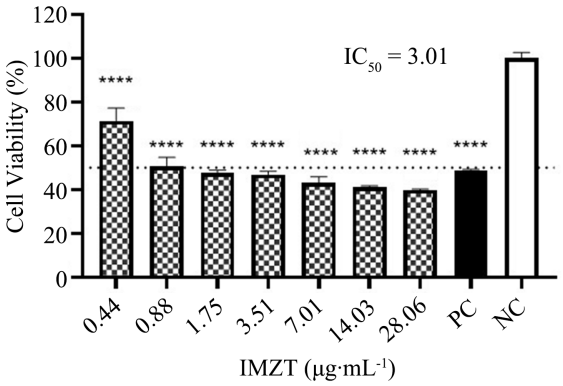
Evaluation of cytotoxic activity on mitochondria was performed using the MTT assay. The values are presented in **Figure 4(a)**. ZF-L cells exposed to IMZT exhibited reduced mitochondrial activity after 24 h of exposure at all concentrations used (0.44 - 28.06 μg/mL) in 28.52%, 49.45%, 52.33%, 53.03%, 56.78%, 58.79%, and 60.16% when compared to the control (NC). The obtained IC_{50} was 3.01 μg/mL.

3.3.3. Neutral Red Uptake Assay

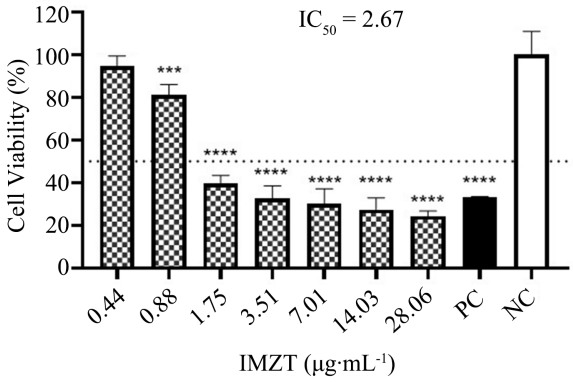
Results of the evaluation of cytotoxic activity on lysosomes are expressed in **Figure 4(b)**. Treatment with IMZT reduced the lysosomal activity of ZF-L cells after 24 h of exposure at concentrations of 0.88 to 28.06 μg/mL in 18.77%, 60.45%, 67.29%, 69.80%, 72.56%, and 75.66% respectively, when compared to the control (NC). The obtained IC_{50} was 2.67 μg/mL

3.3.4. LDH Release Assay

LDH release assay was performed using the supernatant medium of cells exposed to IMZT for 24 hours. It was observed that the herbicide affected the membrane integrity, and cellular leakage a significant increase at concentrations of 3.51 to 28.06 μg/mL in 57.36%, 83.81%, 97.81%, and 101.31% respectively. At concentrations of 14.03 and 28.06 μg/mL, cytotoxicity similar to PC (100%) was observed. The IC_{50} was 1.61 μg/mL (**Figure 4(c)**).



(a)



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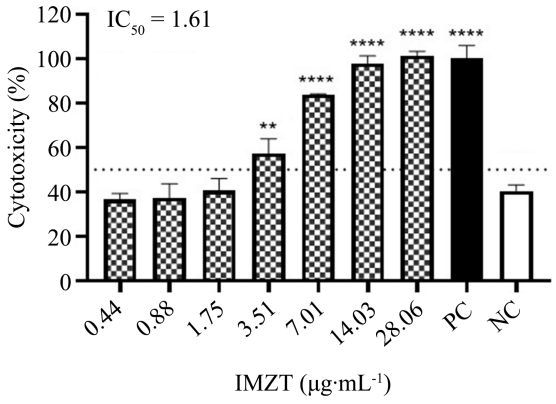


(c)

Figure 4. The cytotoxic effect of IMZT on ZF-L cells was assessed using three different assays: (a) MTT assay, (b) NR uptake assay, and (c) LDH release assay. Cells were treated with IMZT for 24 h at various concentrations. For (a) and (b), H_2O_2 served as the positive control (PC). Data is presented as the percentage of mean ± SD, with the mean of the negative control (NC) set as 100%. In (c), Triton-100× was used as the PC, and the data is expressed as previously mentioned, with the PC set as 100%. Cells treated only with culture medium were used as the negative control (NC) in all assays. To determine the significance of the data, a one-way ANOVA followed by Dunnett's post-hoc test was employed in comparison to the negative control (NC), where $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. The inhibitory concentration value (IC_{50}) is also presented in each assay and was calculated using the non-linear regression method, based on the concentration and percentage of activity.

3.3.5. Estimation of Global IC_{50}

The global IC_{50} values for the various assays are presented in **Table 1**. The median IC_{50} for the herbicide was calculated, obtaining a value of 2.84 ± 0.17 µg/mL.

Table 1. The median IC_{50} value of IMZT was calculated from the IC_{50} of the tests performed (HA, MTT, NR, and LDH).

IMZT	
Essay	[(1)]IC_{50} (µg/mL)
HA	12.75
MTT	3.01
NR	2.67
LDH	1.61
Median IC_{50} [(1)]	2.84 ± 0.17

[(1)]The half-maximal inhibitory concentration.

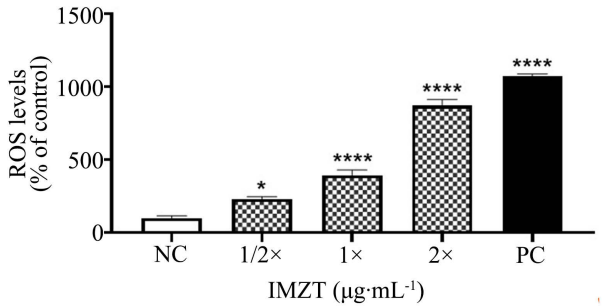
3.3.6. Oxidative Stress Effects on ZF-L Cells

The changes in oxidative stress parameters in ZF-L cells produced after exposure to the formulation containing the IMZT herbicide for 24 hours were evaluated and are described in **Figure 5**. Three different concentrations were tested, cor-

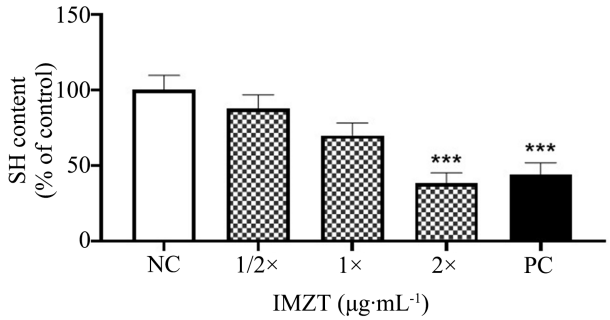
responding to the median IC_{50} values in Table 1. The concentrations used were: 1/2× (1.42 µg/mL), 1× (2.84 µg/mL), and 2× (5.68 µg/mL).

In this study, we observed an increase in ROS production in ZF-L cells induced by IMZT at all evaluated concentrations. When compared to NC, percentage values of 128.22%, 292.47%, and 769.43% were observed, respectively (Figure 5(a)). Additionally, was observed a 61.82% reduction in antioxidant activity non-enzymatic, as determined by the total content of sulfhydryls (Figure 5(b)), at the 2× concentration (5.68 µg/mL) when compared to NC.

Interestingly, regarding the enzymatic activity of antioxidants (SOD and CAT), it was noted that IMZT reduced the activity of these enzymes at all tested concentrations compared to NC (Figure 5(c) and Figure 5(d)). Specifically, SOD activity exhibited reductions of 28.11%, 48.32%, and 64.11%, while CAT activity exhibited reductions of 71.44%, 74.04%, and 91.06%, respectively, at the tested concentrations of 1/2× (1.42 µg/mL), 1× (2.84 µg/mL), and 2× (5.68 µg/mL).



(a)



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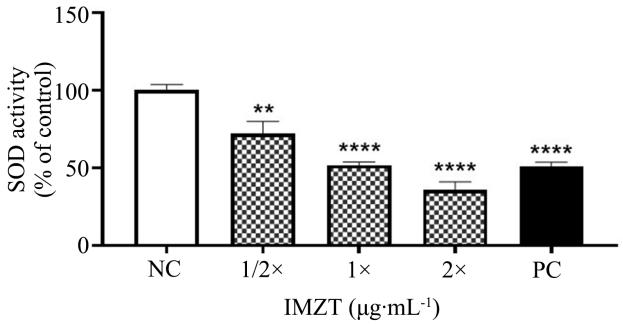

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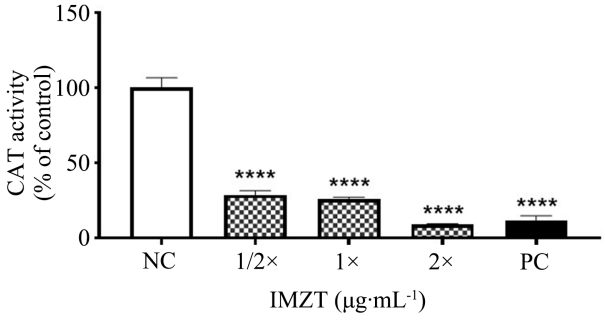

(d)

Figure 5. Oxidative stress parameters in ZF-L cells after 24 h of exposure to median IC_{50} concentrations: 1/2× (1.42 μg/mL), 1× (2.84 μg/mL), and 2× (5.68 μg/mL). (a) Reactive oxygen species (ROS); (b) Total sulfhydryl content; (c) Superoxide dismutase (SOD) activity; (d) Catalase (CAT) activity. Untreated cells were used as the negative control (NC). Data is expressed as a percentage of the NC value. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ when compared with the NC. The positive control (PC) is also shown.

4. Discussion

Most agrochemicals are soluble in water and have high persistence in the soil, which is why they present a high risk for contamination of surface and underground water sources and may cause adverse effects on non-target organisms [1]. Furthermore, smuggled agrochemicals are not registered with the competent bodies, therefore such products may have the composition and concentration of the active ingredient altered and may cause harm to agricultural production, the environment and non-target species [16].

Studies have shown that certain seized contraband agrochemical formulations, analyzed by law enforcement agencies, deviate from the concentrations specified by the manufacturer [15] [16] analyzed illegal agrochemicals and found that the detected active ingredients in the formulations, including IMZT, had quantities lower than those indicated on the labels. In our study, the presence of the active ingredient (IMZT) in the seized herbicide formulation was confirmed by GC-MS, corroborating the findings reported by Berneira *et al.* [15].

We also examined the thermal characteristics of IMZT to understand the thermal decomposition events of this herbicide. In the TGA, IMZT exhibited temperature-induced mass losses. Considering that the formulation in this study is presented in the form of a water-soluble concentrate [15], the mass losses near 100˚C may be associated with the loss of water and/or volatile compounds. Since agrochemicals are complex mixtures containing adjuvants considered inert and not always disclosed by manufacturers, the other events may be related to any additional ingredients comprising the studied herbicide [38] [39].

In the DSC analysis, the physico-chemical transitions occurred in endothermic peaks. However, as not all components of the samples are known, it is not possible to specify the type of transition that occurred in each event. Nevertheless, peaks near 100˚C, with high enthalpy values, may also be related to the evaporation of water, as mentioned earlier. To date, we have not found results in

the literature for comparison with the reported findings here.

Regarding seized agrochemicals, forensic examinations typically include analyses of packaging and labeling, fiscal situations, and, depending on the cases, physico-chemical analyses for qualitative and quantitative determination of formulations [12]. In this sense, assessments of the cytotoxic effects of these agrochemicals seem to be very scarce. Furthermore, to date, *in vitro* assessments of the effects of IMZT and other imidazolinones on animal cells remain limited.

According to the results obtained in this study, *in vitro* assays demonstrated the cytotoxicity of IMZT. The hemolytic activity (HA) assay has been employed to assess the toxic effects of a substance by measuring the degree of hemoglobin release when the erythrocyte membrane is ruptured, and many agrochemicals have been tested using this method [40]. In our study, using this assay, the herbicide IMZT exhibited HA at concentrations ranging from 19.29 to 308.64 μg/mL. In a study reported by Pieniazek *et al.* [41], glyphosate caused hemolysis in human erythrocytes by exposure to 1500 μg/mL for 24 h. Guendouz *et al.* [40] studied the effect of the insecticides abamectin and imidacloprid on human erythrocytes and reported that both increased hemolysis when exposed to concentrations of 400 and 500 μg/mL, respectively. Therefore, our results are consistent with the findings reported above. However, there are no reports of studies assessing the HA of IMZT using this assay.

Regarding the cytotoxicity of IMZT in ZF-L cells, it was possible to assess a toxic effect on these cells, including the reduction of mitochondrial dehydrogenase activity, damage to lysosomes, and compromised cell membrane integrity. No literature data is using IMZT for comparison of results. ZF-L cells exposed to other herbicides such as Roundup Transorb, reduced the mitochondrial activity of ZF-L cells after 6 h of exposure at concentrations of 0.1354 and 0.2708 μg/mL [18]. The same authors observed a reduction in lysosomal integrity after exposure to 0.0677, 0.1354, and 0.2708 μg/L [18]. Lopes *et al.* observed a reduction in mitochondrial activity and lysosomal integrity in ZF-L cells after 24 h of exposure to Roundup at a concentration of 3.25 μg/mL [21]. In another study using ZF-L cells exposed to the metal-insecticide-hesperidin (MgHP), after 24 h of exposure, the insecticide reduced mitochondrial activity at concentrations from 0.001 to 1 μg/mL, while lysosomal integrity decreased at all tested concentrations (0.0001 to 1 μg/mL) [22]. Costa *et al.* [3] evaluated the cytotoxicity of formulations of the herbicides imazethapyr (IMZT, 5 μg/mL) and glyphosate (ATN, 0.5 μg/mL) in their single forms or mixtures M3 (5 μg/mL IMZT + 0.05 μg/mL ATN) in human HepG2 cells. Besides that, the authors demonstrated through the WST-1 assay that there was an increase in cytotoxicity in cells 24 h after exposure to all formulations (IMZT, ATN, and M3) [3]. The same authors demonstrated that IMZT (5 μg/mL), ATN, and M3 decreased cell membrane integrity after 24 h of incubation [3]. Our results are similar to those reported by Costa *et al.* [3], who reported IC_{50} values of 1.65 (24 h), 2.66 (48 h), and 2.01 μg/mL (72 h) in HepG2 cells.

To date, there are few reports on the toxicity of IMZT regarding the induced oxidative stress by this herbicide using vertebrate animal models [6]-[8] [42] and vertebrate cell lines. Concerning the use of cell lines, Soloneski *et al.* [9] demonstrated that IMZT caused DNA damage in Chinese hamster ovary cells (CHO-K1). Therefore, to the best of our knowledge, this is the first study evaluating the *in vitro* effect of oxidative stress induced by IMZT in the ZF-L cell line.

The results of IMZT regarding oxidative stress parameters may reflect an imbalance between the production of reactive oxygen species (ROS) and the action of the antioxidant defense mechanism in the organism, generating a large number of intermediates, including the enzymes CAT, SOD, and non-enzyme –SH [43]. Our results indicated that exposure of ZF-L cells to IMZT significantly increased ROS production and total sulfhydryl content showed a significant decrease in cells exposed to only the highest concentration. Furthermore, IMZT promoted a decrease in the action of CAT and SOD antioxidant defenses at all concentrations studied. Our findings align with those reported by Bonomo *et al.* [23], who observed an increase in ROS levels after exposing ZF-L cells to the metal-insecticide-hesperidin (MgHP) at a concentration of 1 μg/mL (24 h) and a decrease in CAT.

Regarding the observed reductions in SOD and CAT, we suggest that the decrease in their levels may be associated with their action in the defense and protection mechanisms against oxidative stress induced by IMZT in ZF-L cells, with levels depleting over the 24 h exposure period. Regarding the decreased levels of thiol compounds, these may signal a failure of the primary defense system against oxidative stress induced by IMZT in cells. As known, sulfhydryls are part of the thiol group (-SH) in an important non-enzymatic antioxidant system that works together with other enzymes to regulate intracellular metabolism, defending biological structures and functions from the harmful attack of ROS [43] [44].

Our results support the literature data demonstrating that many agrochemicals act by increasing ROS levels in cells and reducing antioxidant defenses [45]. When mild oxidative stress occurs, the defense system is induced to a compensatory response; however, with a drastic increase in ROS, for example, the compensatory processes may be lost. We observed that IMZT significantly increased ROS production in ZF-L cells and, therefore, may have induced oxidative stress responses [46] [47].

5. Conclusions

The results obtained in the present study suggest that the seized formulation of IMZT was capable of inducing cytotoxicity *in vitro* under the conditions and concentrations tested, both in erythrocytes and in the ZF-L cell line. It was also capable of inducing oxidative stress in ZF-L cells, evidenced by the increase in reactive oxygen species (ROS) at all concentrations tested, and by the decrease in the antioxidant system evaluated.

Therefore, taken together, these results indicate the importance of *in vitro* tests for evaluating the toxicity of seized agrochemicals, as they demonstrate the harmful effects that can affect human health and the environment.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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