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Tese

Efeitos das formulações dos agroquímicos imazetapir e quincloraque em linhagem celular de fígado de peixe-zebra (ZF-L) (*Danio rerio*): citotoxicidade e estresse oxidativo

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Resumo

FERRER, Edila Maria Kickhöfel. **Efeitos das formulações dos agroquímicos imazetapir e quincloraque em linhagem celular de fígado de peixe-zebra (ZF-L) (*Danio rerio*): citotoxicidade e estresse oxidativo.** Orientador: Rodrigo de Almeida Vaucher. 2024. 106 f. Tese (Doutorado) – Programa de Pós-Graduação em Bioquímica Bioprospecção. Universidade Federal de Pelotas, Pelotas, 2024.

O Brasil, além de ser um dos países que mais utilizam agroquímicos atendendo às exigências legais, também apresenta indícios de utilização de agroquímicos ilícitos. Diversos estudos apontam os efeitos nocivos destes produtos lícitos, em organismos não-alvos, tanto em ensaios *in vitro* quanto *in vivo*. O objetivo deste estudo foi avaliar a citotocicidade e o estresse oxidativo, *in vitro*, das formulações apreendidas de herbicidas imazetapir (IMZT) e quincloraque (QCR) utilizando linhagem celular hepática ZF-L do peixe zebrafish. O ingrediente ativo nas amostras foi identificado por cromatografia gasosa acoplada à espectrometria de massas (CG-EM). Também foram realizados os testes de estabilidade térmica de análise termogravimétrica (TGA) e a análise de calorimetria exploratória diferencial (DSC). Nas análises térmicas de TGA, foi demonstrado que ocorreu a perda total de massa a 400ºC para as duas amostras e, na de DSC, os herbicidas apresentaram vários eventos de transições físico-químicas. A citotoxicidade, inicialmente, foi avaliada pela atividade hemolítica (AH), utilizando sangue de carneiro desfibrinado (IMZT: 4,82 a 308,64 µg/mL e QCR: 2,63 a 84,82 µg/mL) e, posteriormente, pelos ensaios MTT, vermelho neutro (VN), lactato desidrogenase (LDH), utilizando a linhagem celular hepática do peixe-zebra (ZF-L), expostas aos herbicidas (IMZT: 0,44 a 28,06 µg/mL e QCR: 0,27 a 7,67 µg/mL). O estresse oxidativo foi avaliado pelas determinações das espécies reativas de oxigênio (ERO), do teor total de sulfidrilas (SH) e das atividades antioxidantes da superóxido dismutase (SOD), da catalase (CAT) também em células ZF-L, expostas a 1/2x, 1x e 2x da mediana dos IC_{50} dos ensaios de citotoxicidade. O período de exposição das células ZF-L aos herbicidas foi de 24 horas. No ensaio hemolítico, os eritrócitos foram afetados nas concentrações mais elevadas tanto para o IMZT (19,29 a 308,64 µg/mL) quanto ao QCR (21,08 a 84,82 µg/mL) e apresentaram IC_{50} de 12,75 µg/mL e 19,83 µg/mL, respectivamente. Nos ensaios de MTT, NR e LDH, ambos os herbicidas afetaram as organelas mitocondrial e lisossomal e a integridade da membrana plasmática. As IC_{50} do IMZT foram de 3,01, 2,67 e 1,61 µg/mL, respectivamente, e para o QCR foram de 1,78, 5,13 e 0,95 µg/mL, respectivamente. A partir dessas concentrações de IC_{50}, foi estimado o valor da mediana que correspondeu a IC_{50} de 2,84 µg/mL (IMZT) e 3,46 µg/mL (QCR). Os resultados das IC_{50} das medianas foram utilizados nos ensaios de estresse oxidativo. Tanto o IMZT quanto o QCR aumentaram a produção de ERO e promoveram alterações no sistema antioxidante do teor total de SH, da SOD e da CAT. Até onde sabemos, este é o primeiro estudo que reporta o efeito citotóxico *in vitro* e do estresse oxidativo induzidos por formulações apreendidas de herbicidas contendo IMZT e QCR, na linhagem celular ZF-L. Em conjunto, esses resultados indicam a importância de ensaios *in vitro* para a avaliação da toxicidade de agroquímicos, pois demonstram os efeitos nocivos que podem afetar a saúde humana e o meio ambiente.

Palavras-chave: *in vitro*; viabilidade celular; *zebrafish*; hepatócitos; dano oxidativo.

Abstract

FERRER, Edila Maria Kickhöfel. **Effects of formulations of the agrochemicals imazethapyr and quinchlorac on zebrafish liver cell line (ZF-L) (*Danio rerio*): cytotoxicity and oxidative stress.** Advisor: Rodrigo de Almeida Vaucher. 2024. 106 f. Thesis (Doctorate) – Biochemistry and Bioprospection Post-Graduate Program, Food, Pharmaceutical and Chemical Sciences Center. Federal University of Pelotas, Pelotas, Pelotas, 2024.

Brazil, in addition to being one of the countries that uses the most agrochemicals in compliance with legal requirements, also shows evidence of the use of illicit agrochemicals. Several studies point to the harmful effects of these legal products on non-target organisms, both *in vitro* and *in vivo*. The objective of this study was to evaluate the cytotoxicity and oxidative stress, *in vitro*, of the seized formulations of herbicides imazethapyr (IMZT) and quinclorac (QCR) using the zebrafish liver cell line ZF-L. The active ingredient in the samples was identified by gas chromatography coupled with mass spectrometry (GC-MS). Thermogravimetric analysis (TGA) thermal stability tests and differential scanning calorimetry (DSC) analysis were also carried out. In the TGA thermal analyses, it was demonstrated that total mass loss occurred at 400 ºC for both samples, and, in the DSC analysis, the herbicides presented several physicochemical transition events. Cytotoxicity was initially assessed by hemolytic activity (AH), using defibrinated sheep blood (IMZT: 4.82 to 308.64 µg/mL and QCR: 2.63 to 84.82 µg/mL) and, subsequently, by MTT, neutral red (VN), lactate dehydrogenase (LDH) assays, using the zebrafish liver cell line (ZF-L), exposed to herbicides (IMZT: 0.44 to 28.06 µg/mL and QCR: 0.27 to 7.67 µg/mL). Oxidative stress was evaluated by determining reactive oxygen species (ROS), total sulfhydryl content (SH) and antioxidant activities of superoxide dismutase (SOD), catalase (CAT) also in ZF-L cells, exposed to 1/2x, 1x and 2x of the median IC_{50} of cytotoxicity assays. The period of exposure of ZF-L cells to herbicides was 24 hours. In the hemolytic assay, erythrocytes were affected at the highest concentrations for both IMZT (19.29 to 308.64 µg/mL) and QCR (21.08 to 84.82 µg/mL) and presented an IC_{50} of 12.75 µg/mL and 19.83 µg/mL, respectively. In the MTT, NR, and LDH assays, both herbicides affected mitochondrial and lysosomal organelles and the integrity of the plasma membrane. The IC_{50} for IMZT was 3.01, 2.67, and 1.61 µg/mL, respectively, and for QCR theywere 1.78, 5.13, and 0.95 µg/mL, respectively. From these IC_{50} concentrations, the median value was estimated, corresponding to IC_{50} of 2.84 µg/mL (IMZT) and 3.46 µg/mL (QCR). The IC_{50} results of the medians were used in oxidative stress assays. Both IMZT and QCR increased the production of ROS and promoted changes in the antioxidant system in the total content of SH, SOD and CAT. To our knowledge, this is the first study that reports the *in vitro* cytotoxic effect and oxidative stress induced by seized formulations of herbicides containing IMZT and QCR, in the ZF-L cell line. Taken together, these results indicate the importance of *in vitro* tests for evaluating the toxicity of

agrochemicals, as they demonstrate the harmful effects that can affect human health and the environment.

Keywords: *in vitro*; cell viability; zebrafish; hepatocytes; oxidative damage.

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Lista de Abreviaturas e Siglas

ALS	Acetolactato sintase
ANVISA	Agência Nacional de Vigilância Sanitária
BCRJ	Banco de Células do Rio de Janeiro
CAT	Catalase
CG-EM	Cromatografia gasosa acoplada à espectrometria de massas
CL_{50}	Concentração letal 50%
DMEM	*Dulbecco's Modified Eagle Medium*
DMSO	Dimetilsulfóxido
DNA	Ácidodesoxirribonucleico
DSC	Calorimetria exploratória diferencial
DT_{50}	Tempo para 50% de dissipação = "meia-vida" da bioatividade
EO	Estresse oxidativo
ERO	Espécies reativas de oxigênio
FISPQ	Ficha de Informações de Segurança de Produtos Químicos
HEPES	*N-(2-hydroxyethyl)-Piperazine ethane sulfonic acid*
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
IC_{50}	Concentração inibitória capaz de diminuir a viabilidade celular em 50%
IMZT	Imazetapir
IMZT-R	Enantiômero R (+), latim *rectus*
IMZT-S	Enantiômero S (-), latim *sinister*
L-15	*Leibovitz's L-15 Medium*
LDH	Lactato desidrogenase (*lactate dehydrogenase*)
MAPA	Ministério da Agricultura e Pecuária
MTT	Metil-tiazolil-tetrazólio (Brometo de 3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazólio)
$O_2^{\bullet -}$	Ânion superóxido
$OH^{\bullet}$	Radical hidroxila
PBS	Tampão salino fosfato (*Phosphate-buffered saline*)
QCR	Quincloraque
RPM	Rotações por minuto

SFB	Soro fetal bovino
SH	Sulfidrila
SOD	Superóxido dismutase
TGA	Análise termogravimétrica
VN	Vermelho neutro (2-amino-3-metil 7dimetil-amino-cloreto de fenazina)
ZF-L	*Zebrafish-Liver* - Linhagem de células hepáticas de *zebrafish* (*Danio rerio*)

Sumário

1 Introdução

Agroquímicos, em sua maioria, são substâncias químicas sintéticas que visam ao combate ou controle de pragas que prejudicam a produção de alimentos (TUDI *et al.,* 2021; SOUZA *et al*., 2023). No entanto, é necessário considerar que, modelos agrícolas que fazem uso intensivo e o uso indiscriminado de agroquímicos, podem contribuir para a contaminação, não só dos alimentos, como também do solo, do ar e da água, gerando diversos problemas a organismos não-alvos, tanto aos invertebrados quanto aos vertebrados, incluindo humanos e peixes (LUSHCHAK *et al*., 2018; DAAM *et al.,* 2019; TUDI *et al.,* 2022; SOUZA *et al*., 2023).

Dependendo de diversos fatores como as propriedades físicas e químicas, tipo de formulação, modo de aplicação e das características do receptor hídrico, essas substâncias podem contaminar sistemas aquáticos superficiais e, por meio de percolação da água no solo, podem chegar às águas subterrâneas (LOPES; ALBUQUERQUE, 2018; SYAFRUDIN *et al*., 2021). Neste sentido, estudos apontam que resíduos de diversas classes de agroquímicos, incluindo herbicidas, são encontrados em águas potáveis, em concentrações bastante significativas (CALDAS *et al.,* 2019; SOUZA *et al*., 2020; PANIS *et al*., 2022a) e, mesmo em concentrações mínimas, podem apresentar riscos à saúde humana (SYAFRUDIN *et al*., 2021; PANIS *et al*., 2022a).

Assim como acontece com a maioria dos agroquímicos, apenas uma pequena parte dos herbicidas aplicados na agricultura atinge o alvo desejado. A maior parte pode resultar em acúmulo de resíduos no meio ambiente, e quando as áreas agrícolas são próximas a corpos d'água, os sistemas aquáticos podem ser os mais afetados (MAGDALENO *et al*., 2015; FATHY *et al*., 2019; BROVINI *et al*., 2021; PÉREZ-IGLESIAS *et al*., 2023). Normalmente, os agroquímicos estão presentes em baixas concentrações nesses ambientes, e nem sempre resultam em mortalidade imediata dos organismos não-alvos, tais como peixes. No entanto, podem causar efeitos nocivos em diferentes tipos de células e atingir funções celulares distintas (GOULART; BOYLE; SOUZA, 2015).

Em peixes, por exemplo, herbicidas podem provocar diversos distúrbios, incluindo o sistema imunológico, neurológico, respiratório, reprodutivo, hepático entre outros (AMENYOGBE *et al*., 2021; RIBEIRO *et al*. 2022). Neste sentido, o fígado, pelo fato de ser o principal órgão envolvido na metabolização de diversos

xenobióticos, antioxidantes relacionados ao estresse oxidativo, incluindo os enzimáticos superóxido dismutase (SOD) e catalase (CAT) fazem parte dos principais alvos investigados nestes animais (RIBEIRO *et al*. 2022; SALEH *et al*., 2022; SILVA *et al*., 2023).

Segundo a Organização das Nações Unidas para Agricultura e Alimentação (FAO), em 2021, em termos globais, foram utilizados aproximadamente 3,53 milhões de toneladas de agroquímicos para uso agrícola. Sendo que ao Brasil, corresponde um consumo próximo a 720 mil toneladas (FAO, 2021).

O Brasil é considerado um dos países que mais utilizam agroquímicos e, além de ser um dos maiores consumidores desses produtos comercializados de forma legal, também ocupa uma posição bastante preocupante no que se refere à utilização de produtos obtidos de forma ilícita, resultando em quantidades significativas de apreensões, pelas forças de segurança nacional, em várias regiões do Brasil (FRAGA *et al*., 2016; LEMOS; CARVALHO; ORTIZ, 2018). Formulações, incluindo imazetapir (IMZT) e quincloraque (QCR) são alguns dos agroquímicos apreendidos no sul do país (LEMOS; CARVALHO; ORTIZ, 2018; BERNEIRA *et al.,* 2020).

Vários estudos apontam que agroquímicos apreendidos, nem sempre apresentam a composição química ou a concentração do ingrediente ativo da formulação, conforme descrito no rótulo (CROCOLI; ORTIZ; MOURA*,* 2019; CROCOLI; ORTIZ; MOURA*,* 2020; BERNEIRA *et al.*, 2020), portanto, podem, além de trazer prejuízos à produção agrícola, ser prejudiciais à saúde humana e ao meio ambiente (LEMOS; CARVALHO; ORTIZ, 2018; SANT'ANA *et al.*, 2019; BAYOUMI, 2021).

Diversos estudos demonstraram a toxicidade de várias classes de agroquímicos, tanto em modelos *in vivo* quanto *in vitro* (GONÇALVES *et al*., 2020; KATAGI, 2020), contribuindo com informações preliminares sobre a toxicidade desses compostos. No entanto, nos agroquímicos apreendidos, normalmente são realizados diversos procedimentos periciais que compreendem análises de embalagem e rotulagem, situação fiscal e, dependendo da situação, a identificação química do ingrediente ativo (LEMOS; CARVALHO; ORTIZ, 2018; BERNEIRA *et al*., 2020; CROCOLI; ORTIZ; MOURA, 2020). Além disso, não foram encontrados relatos quanto a análises de citotoxicidade de formulações apreendidas.

Até o presente momento, avaliações *in vitro* sobre os efeitos dos herbicidas IMZT e QCR em células animais continuam limitadas. Ainda mais escassas àquelas associadas a avaliações dos efeitos citotóxicos de agroquímicos contrabandeados, ou de produção ilícita, apreendidos por órgãos federais institucionais do país. Contudo, até onde sabemos, este é o primeiro estudo realizado com esses tipos de herbicidas utilizando a linhagem celular hepática *Zebrafish-liver* (ZF-L) para avaliar a citotoxicidade e o estresse oxidativo. Esta linhagem celular é proveniente de um vertebrado aquático (*Zebrafish – Danio rerio*) e seu uso está bem descrito na literatura, demonstrando sua importância na identificação de risco toxicológico de agroquímicos para organismos aquáticos (GOULART; BOYLE; SOUZA, 2015; LOPES; SANDRINI; SOUZA, 2018; BONOMO *et al*., 2019; BONOMO *et al*., 2020).

Diante do exposto, a relevância deste estudo consiste no fato de que a avaliação toxicológica, *in vitro,* pode complementar os procedimentos de análises periciais de agroquímicos apreendidos pelos órgãos de segurança pública, contribuindo para o conhecimento sobre a toxicidade destes, em modelos celulares.

2 Objetivos

2.1 Objetivo Geral

O objetivo deste estudo foi avaliar a citotoxicidade e o estresse oxidativo de formulações apreendidas dos herbicidas imazetapir (IMZT) e quincloraque (QCR) em células da linhagem hepática ZF-L de *zebrafish*.

2.2 Objetivos Específicos

- Confirmar a presença dos princípios ativos nas formulações dos herbicidas IMZT e QCR e caracterizar termicamente;

- Investigar a atividade hemolítica das formulações dos herbicidas IMZT e QCR e estimar a IC_{50};

- Determinar a citototoxicidade das formulações dos herbicidas IMZT e QCR em hepatócitos de *D. rerio* (linhagem ZF-L);

- Avaliar o estresse oxidativo causado pelas formulações dos herbicidas IMZT e QCR em hepatócitos de *D. rerio* (linhagem ZF-L).

3 Referencial teórico

3.1 Agroquímicos

Agroquímicos, de um modo geral, são formulações que possuem em sua composição ingredientes químicos ou biológicos que atuam para o combate ou controle de organismos, incluindo animais e vegetais, que causam danos à produção agrícola (SYAFRUDIN *et al*., 2021; TUDI *et al.,* 2021. Sem o seu uso, as perdas representariam aproximadamente 78% na produção de frutas, 54% na produção de vegetais e de 32% na produção de cereais (TUDI *et al*., 2021).

O uso destes produtos teve um aumento expressivo a partir da introdução de agroquímicos orgânicos sintéticos na década de 1940 (DAYAN, 2019; GAINES *et al*., 2020). Também são chamados de pesticidas, produtos fitossanitários, defensivos agrícolas entre outras denominações (DE MORAES, 2021), sendo que, a nomenclatura oficial adotada no Brasil é agrotóxico (BRASIL, 1989; BRASIL, 2023).

De acordo com o organismo-alvo que controlam, os agroquímicos são classificados em herbicidas, inseticidas, fungicidas entre outros (LUSHCHAK *et al*., 2018; SYAFRUDIN *et al*., 2021; TUDI *et al.,* 2021). Também podem ser classificados de acordo com a estrutura química do ingrediente ativo ou com o grau de toxidade, neste caso, relacionados aos efeitos à saúde humana e ao meio ambiente (SHARMA; SHARMA; CHOPRA, 2020; TUDI *et al*., 2021; SOUZA *et al*., 2023).

No Brasil, em 2019, foi publicada pela Agência Nacional de Vigilância Sanitária (ANVISA), uma reclassificação para os agroquímicos com base nos padrões do Sistema Globalmente Harmonizado de Classificação e Rotulagem de Produtos Químicos (*Globally Harmonized System of Classification and Labelling of Chemicals* – GHS), sendo considerado um novo marco regulatório (ANVISA, 2019; NUNES *et al*., 2021).

Conforme mostrado na Tabela 1, os agroquímicos foram distribuídos em seis categorias e receberam cores para determinação da toxicidade (ANVISA, 2019; NUNES *et al*., 2021). Além disso, o sistema de classificação de toxicidade do agroquímico leva em conta os riscos à saúde humana, considerando a toxicidade oral e dérmica aguda (DL_{50}) para ratos (Tabela 1), uma vez que estas determinações são procedimentos padrão em toxicologia (WHO, 2020).

Tabela 1 – Reclassificação da toxicidade de agroquímicos no Brasil emitida em 2019.

			DL_{50}[(1)] para o rato (mg/kg de p. c.[(2)])	
Categoria	**Toxicidade**	**Risco**	**Oral**	**Dérmica**
1 – Faixa vermelha	Produto Extremamente Tóxico	Fatal se ingerido, em contato com a pele ou inalado	<5	<50
2 – Faixa vermelha	Produto Altamente Tóxico	Fatal, dependendo da exposição	5–50	50–200
3 – Faixa amarela	Produto Moderadamente Tóxico	Causa intoxicação se ingerido, em contato com a pele ou inalado	50–2000	200–2000
4 – Faixa azul	Produto Pouco Tóxico	Nocivo se ingerido, em contato com a pele ou inalado	Acima de 2000	Acima de 2000
5 – Faixa azul	Produto Improvável de Causar Dano Agudo	Pode ser perigoso se ingerido, em contato com a pele ou inalado	5000 ou superior	
Não classificado – Faixa verde	Produto Não Classificado	Sem riscos ou recomendações		

[(1)]Estimativa estatística de mg de substância tóxica por kg de peso corporal necessária para matar 50% de uma população de animais-teste; [(2)]Peso Corporal.
Fonte: Adaptado de ANVISA, 2019; WHO, 2020; Nunes *et al*., 2021.

Cabe mencionar que os agroquímicos possuem em suas formulações, além do ingrediente ativo, adjuvantes, considerados inertes e nem sempre revelados pelos fabricantes, tais como surfactantes, agentes antiespumantes entre outros, para facilitar a absorção e estabilidade do ingrediente ativo e assim melhorar a ação pesticida (MESNAGE *et al*., 2014; MESNAGE; ANTONIOU, 2018). No entanto, apesar de consideradas inertes, a toxicidade de algumas destas substâncias é conhecida e estudos demonstraram que podem ser mais tóxicas que o ingrediente ativo da formulação, em organismos não-alvos (MESNAGE; BERNAY; SÉRALINI, 2013; MESNAGE; ANTONIOU, 2018).

Atualmente, quanto à classificação ambiental, os agroquímicos estão divididos em quatro classes: Classe I - Produto Altamente Perigoso ao Meio Ambiente; Classe II – Produto Muito Perigoso ao Meio Ambiente; Classe III – Produto Perigoso ao Meio Ambiente; e Classe IV – Produto Pouco Perigoso ao Meio Ambiente. Essa avaliação realizada pelo Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), teve como base alguns critérios relacionados às características do produto, como as propriedades físico-químicas e a ecotoxicidade para os organismos não-alvos, capacidade de bioacumulação, persistência no meio ambiente e o comportamento no solo, no ar ou na água (IBAMA, 2022).

No Brasil, o uso de agroquímico teve a sua regulação pela Lei nº 7.802/89 (BRASIL, 1989); a partir do final do ano de 2023, passou a ser regulamentado pela Lei nº 14.785/2023 (BRASIL, 2023). A regulamentação e a aprovação de novas formulações de agroquímicos, normalmente é um processo muito complexo e pode se estender por vários anos (IDESF, 2019). Mesmo assim, no Brasil, existem registros de, aproximadamente, 1.159 formulações comerciais de herbicidas (MAPA/AGROFIT, 2024).

Os agroquímicos mais consumidos no mundo são os herbicidas, seguido por inseticidas e fungicidas (SHARMA; SHARMA; CHOPRA, 2020; LEOCI; RUBERTI, 2021). Os herbicidas representam aproximadamente 60% do total dos agroquímicos utilizados mundialmente. São essenciais, principalmente, na produção agrícola em larga escala, para o controle e a eliminação de plantas indesejáveis (DAYAN, 2019; FATHY *et al*., 2019; GAINES *et al*., 2020). Segundo o Sindicato Nacional da Indústria de Produtos para Defesa Vegetal (Sindiveg), em 2023, foram consumidas, aproximadamente, 1.424,588 toneladas de agroquímicos na produção agrícola brasileira. Destes, 47% correspondem ao consumo de herbicidas (SINDIVEG, 2024).

Devido à relevância mundial no setor agrícola, o Brasil é bastante dependente da utilização de agroquímicos, e isso tem despertado o interesse tanto de empresas para o comércio lícito como também ao comércio destes produtos, através do contrabando ou de sua produção ilícita (clandestina) (SANT'ANA *et al*., 2019; GABOARDI; CANDIOTTO; PANIS, 2023). Esta atividade pode ser caracterizada como crime de contrabando ou de falsificação ou, ainda, como crime contra o meio ambiente (LEMOS; CARVALHO; ORTIZ, 2018; CROCOLI; ORTIZ; MOURA, 2019; IDESF, 2019).

No Brasil, de acordo com o Instituto de Desenvolvimento Econômico e Social de Fronteiras (IDESF), aproximadamente 25% dos agroquímicos utilizados são de origem ilegal (IDESF, 2021). E para o combate ao comércio ilícito no território nacional, ocorre a atuação em conjunto das forças de segurança do país, cujas operações resultam em apreensões de quantidades bastante significativas de agroquímicos (LEMOS; CARVALHO; ORTIZ, 2018; IDESF, 2021). Entre 2007 e 2018, órgãos federais apreenderam, em vários pontos do país, aproximadamente 1,3 mil cargas de agroquímicos contrabandeados ou com suspeita de serem falsificados (DE MORAES, 2022). E, entre janeiro de 2018 e fevereiro de 2021, foram apreendidas aproximadamente 215 toneladas, sendo que, 6,7% correspondem ao estado do Rio Grande do Sul (IDESF, 2021).

As motivações para a obtenção destes produtos a partir do comércio ilícito podem estar associadas, principalmente, aos altos custos dos produtos comerciais e às elevadas taxas de importação aplicadas a estes, que desfavorecem a obtenção de lucro na produção agrícola (CROCOLI; ORTIZ; MOURA, 2020; BERNEIRA *et al*., 2020; CARVALHO *et al*., 2023). Além disso, muitos estados do território brasileiro fazem fronteira terrestre com outros países, como o estado do Rio Grande do Sul, que faz fronteira com o Uruguai, por exemplo. Neste país, não é necessária a prescrição agronômica para aquisição de alguns destes produtos (DORFMAN; FRANÇA; SOARES, 2014; IDESF, 2019).

No entanto, em se tratando de produtos ilícitos, estes não passam por testes e pela aprovação de agências governamentais quanto ao controle sanitário ou ambiental, podendo conter concentração do ingrediente ativo ou substâncias diferentes das descritas no rótulo (CROCOLI; ORTIZ; MOURA, 2019; CROCOLI; ORTIZ; MOURA, 2020; BERNEIRA *et al.*, 2020), e assim, representar maior risco ao meio ambiente e à saúde humana, principalmente, do agricultor (FREZAL; GARSOUS, 2020; BAYOUMI, 2021). Também é necessário considerar que, muitas vezes, as pessoas que manuseiam estas formulações, desconhecem ou não seguem os protocolos de segurança e de proteção individual, aumentando as chances de intoxicações agudas ou crônicas (PINTO *et al*., 2020; PANIS *et al*., 2022b).

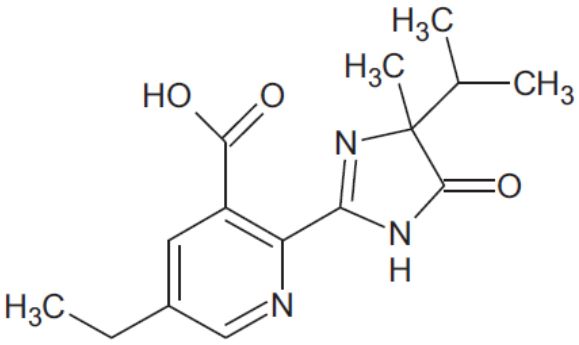
Ainda, com relação a agroquímicos ilícitos, outro fator que representa riscos de contaminação ambiental, está relacionado à disposição das embalagens vazias. Embalagens contaminadas com resíduos, quando descartadas de forma irregular na

natureza, os resíduos podem contaminar o solo e as águas, tanto as superficiais quanto as subterrâneas (IDESF, 2019; MELLO; SCAPINI, 2016; BAYOUMI, 2021).

3.1.1 Imazetapir

O herbicida IMZT [ácido(RS)-5-etil-2-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il)nicotínico] pertence ao grupo químico das imidazolinonas, a fórmula bruta é $C_{15}H_{19}N_3O_3$ (ANVISA, 2022) e sua molécula é formada por amina aromática heterocíclica (Figura 1) (KOUTROS *et al*., 2016). Possui ação seletiva sistêmica e seu mecanismo de ação consiste na supressão da síntese de aminoácidos de cadeia ramificada (valina, leucina e isoleucina), pela inibição da enzima acetolactato sintase (ALS), interrompendo o crescimento da planta e resultando na sua eliminação (SOLONESKI *et al*., 2017; GAINES *et al*., 2020; YAO *et al*., 2020).

Figura 1 - Fórmula estrutural do imazetapir.



Fonte**:** LIMAN; CIGERCI, ÖZTÜRK, 2015.

Pelo fato de apresentar amplo espectro no controle de plantas daninhas, formulações contendo IMZT são utilizadas em diversos países (PASHA, 2013; MAGDALENO *et al*., 2015; KOUTROS *et al.,* 2016; SOLONESKI *et al*., 2017; CALDAS *et al*., 2019; YAO *et al*., 2020; BOURDINEAUD, 2022; PÉREZ-IGLESIAS *et al*., 2023). No Brasil, é indicado para o controle pré ou pós-emergência de plantas infestantes, dependendo do tipo de cultura, incluindo, amendoim, arroz, soja, feijão e pastagens (ANVISA, 2022).

A degradação do IMZT no solo é atribuída, principalmente, à atuação microbiana, podendo esta biotransformação ser afetada por diversos fatores, incluindo textura do solo, condições ambientais e práticas agrícolas, fazendo deste herbicida altamente persistente no solo, com meia-vida (DT_{50}) com uma variação de

7 a 513 dias (KAUR; KAUR, 2022). Além disso, a alta solubilidade em água (1.400 mg/L) e o baixo coeficiente de adsorção no solo, o torna um potencial contaminante de águas superficiais e subterrâneas, gerando grande preocupação ambiental quando usado de forma intensiva, podendo afetar espécies não vegetais (SOLONESKI et al., 2017; KAUR; KAUR, 2022).

O IMZT é comercializado no Brasil, tanto na formulação de concentrado solúvel (SL) quanto na forma de granulados dispersíveis em água (WG) (MAPA/AGROFIT, 2024). Este herbicida tem um átomo de carbono assimétrico resultando em dois enantiômeros. Apesar do R(+) IMZT ser aproximadamente 8 a 10 vezes mais potente que o S(-) IMZT na inibição da ALS, é comercializado como uma mistura racêmica, resultando na liberação igual de ambos enantiômeros no meio ambiente após o uso (YAO *et al*., 2020).

De acordo com as bulas de alguns produtos comerciais listados na Tabela 2, a classificação toxicológica varia da Categoria 4 (Produto pouco tóxico) a Categoria 5 (Produto improvável de causar dano agudo). No entanto, quanto à classificação do Potencial de Periculosidade Ambiental, está enquadrado na Classe III (Produto Perigoso ao meio ambiente) (MAPA/AGROFIT, 2024). De acordo com os dados fornecidos pelas Fichas de Informações de Segurança de Produtos Químicos (FISPQ), a toxicidade aguda em ratos, assim como a ecotoxicidade (CL_{50}-96 horas) em peixes, pode variar de acordo com o fabricante.

Conforme mostrado na Tabela 2, a CL_{50}-96 horas, para a mesma espécie de peixe (*D. rerio*) varia de 0,73 a 672,60 mg/L. Neste sentido, Costa *et al*. (2022) reportaram um valor de CL_{50}-96 horas de 71,60 mg/L de uma formulação de IMZT para o peixe-zebra nos estágios iniciais, através do FET test – *Fish EmbryoToxicity Test*. Também foram apresentadas concentrações letais de formulações contendo o ingrediente ativo IMZT em outras espécies de peixes (*Colossoma macropomum, Ictalurus punctatus, Onchorhynchus mykiss, Lepomis macrochirus*), cujas CL_{50}-96 horas variaram de 184,15 mg/L a 420 mg/L (ARANHA, 2013; CARVALHO *et al*., 2019).

Diversos estudos relatam efeitos subletais em espécies não-alvos, incluindo peixes, como inibição da enzima acetilcolinesterase (AChE) em peixes da espécie *Oreochromis mossambicus* após exposição a uma formulação comercial de IMZT (PASHA, 2013), e na espécie *Cyprinus carpio* após exposição a uma formulação comercial contendo imazetapir e imazapic, Only® (MORAES *et al*., 2011). Neste

mesmo estudo, os autores também demonstraram que ocorreram alterações em alguns parâmetros de estresse oxidativo, incluindo a enzima catalase (CAT) (MORAES *et al*., 2011).

Tabela 2 – Ecotoxicidade em peixes e efeitos agudos em ratos de laboratório, de alguns produtos à base de imazetapir comercializados no Brasil, na formulação de concentrado solúvel.

	Ecotoxicidade	**Efeitos agudos em ratos**		
Nome do produto (comercial)	**CL_{50}[(2)] (96h) (*Brachy*) *D. rerio*** (mg/L)	**DL_{50}[(3)] oral** (mg/kg p.c.)	**DL_{50} dérmica** (mg/kg p.c.)	**CL_{50} (4h)[(4)] inalatória** (mg/L)
Hummer® (10,60% m/v)[(1)]	0,73	>2000	>4000	>4,948
Imazetapir CCAB 106 SL® (10,60% m/v)	28,28	>5000	>4000	>4,948
Imazetapir Nortox Plus® (10,60% m/v)	17,12	>2000	>2000	>20,0
Pistol® (10,60% m/v)	35,36	>2000	>4000.	>3,12
Vezir® (10,60% m/v)	672,60	>3000	>4000	>21,25
Zethapyr® (10,60% m/v)	411,47	>3000	>4000	>20,0

[(1)]Concentração do ingrediente ativo; [(2)]Concentração Letal Média capaz de causar a mortalidade da metade dos organismos; [(3)]Dose Letal Média capaz de causar mortalidade da metade dos organismos; [(4)](>10,0 - ≤20,0 mg/L/4 h). Fonte: MAPA/AGROFIT, 2024; FISPQ – Internet.

Além disso, Golombieski *et al.* (2016) avaliaram a toxidade aguda de uma formulação comercial contendo os herbicidas imidazolinonas imazapir e imazapic, em peixes da espécie *Rhamdia quelen,* cujos resultados demonstraram que a formulação apresentou efeito tóxico agudo sobre a homeostase, afetando diversas funções vitais, como as defesas imunológicas, metabolismo e neurotransmissão.

Também, estudos apresentados por Costa *et al*. (2022) demonstraram, em um teste de toxidade utilizando embriões de peixe-zebra (*D. rerio*), expostos a concentrações de 0,5-100 µg/mL da formulação do IMZT, que este agroquímico foi capaz de induzir letalidade, bem como inibir a inflação da bexiga natatória. O herbicida também mostrou uma redução na porcentagem de fluorescência em células provenientes das larvas, coradas com o corante Hoechst 33342, sugerindo a perda de DNA.

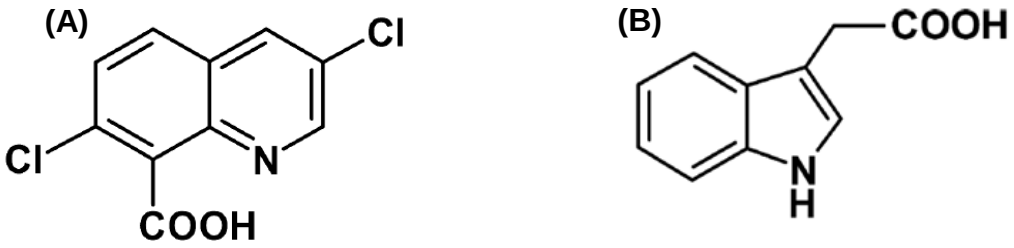
Ainda que, herbicidas da classe das imidazolinonas, como o imazapir, imazaquim e o imazetapir sejam considerados de baixa toxidade aguda para mamíferos (WHO, 2020), Yao *et al*. (2020) demonstraram que o IMZT possui toxicidade enantiosseletiva em camundongos. A exposição ao S(-) IMZT causou interferências mais fortes no sistema de defesa antioxidante em comparação ao R(+) IMZT (YAO *et al*., 2020). Além disso, pessoas expostas a esse tipo de herbicida estão propensas a desenvolver câncer de bexiga e de cólon, conforme relatado por Koutros *et al.* (2016). Neste mesmo sentido, Soloneski *et al*. (2017), demonstraram a genotoxidade induzida pelo ingrediente ativo IMZT e sua formulação, em células de ovário de hamster chinês (CHO-K1) expostas a uma concentração de 0,1 µg/mL.

3.1.2 Quincloraque (Quinclorac)

O herbicida QCR (ácido 3,7-dicloroquinolina-8-carboxílico) pertence ao grupo químico do ácido quinolinocarboxílico e possui fórmula bruta $C_{10}H_5Cl_2NO_2$ (ANVISA, 2022). É um composto clorado formado por um grupo carboxila e um anel aromático (Figura 2-A), altamente persistente no ambiente e, dependendo das características físico-químicas dos solos, os valores de meia-vida (DT_{50}) variam de 57,7 a 266,5 dias (VIDAL; BÁEZ; SALAZAR, 2021). Apesar de sua baixa solubilidade em água (0,065 mg/L a 20 °C) (MENEZES *et al*., 2014), o QCR é classificado como um potencial contaminante de águas subterrâneas e superficiais (VIDAL; BÁEZ; SALAZAR, 2021).

O QCR pertence à classe dos herbicidas que possuem o mecanismo de ação pela mimetização de auxinas (GROSSMANN, 2010; BUSI *et al*., 2018; GAINES *et al*. 2020). Também são conhecidos como herbicidas hormonais, reguladores de crescimento ou auxinas sintéticas por possuírem similaridade estrutural com a auxina natural das plantas, o ácido indol-3-acético (AIA) (Figura 2-B) (GROSSMANN, 2010; GAINES *et al*., 2020).

Figura 2 – Fórmula estrutural do quincloraque (A) e do ácido indol-3-acético (AIA) (B).



Fonte: GROSSMANN, 2010.

É um herbicida amplamente utilizado em diversos países para o controle de ervas daninhas (GROSSMANN, 2010; FATHY *et al.,* 2019; VIDAL; BÁEZ; SALAZAR, 2021). No Brasil, é indicado para controle pós-emergente de várias espécies de plantas daninhas na cultura de arroz irrigado (ANVISA, 2022). É comercializado no país, na formulação de pó solúvel em água (WP). Devido a sua alta mobilidade e persistência, o QCR é um potencial contaminante ambiental tanto de solos e quanto de águas, podendo ser nocivo a organismos não-alvos (VIDAL; BÁEZ; SALAZAR, 2021).

A classificação toxicológica do QCR está relacionada à categoria 5, sendo considerado um produto improvável de causar dano agudo. No entanto, é considerado perigoso ao meio ambiente (Classe III) (MAPA/AGROFIT, 2024). Sua toxicidade aguda em ratos e a ecotoxicidade (CL_{50}-96 horas) em peixes, é mostrada na Tabela 3 conforme dados fornecidos na Ficha Informações de Segurança de Produtos Químicos (FISPQ) (FISPQ)/BASF/2021.

Também foram encontradas concentrações letais de formulações contendo o ingrediente ativo QCR em outras espécies de peixes. Em *D. rerio* a CL_{50}-48 horas foi de 300 mg/L (NAKAGOME; NOLDIM.; RESGALLA, 2007) e em alevinos de *R. quelen* a CL_{50}-96 horas foi de 395 mg/L (MIRON *et al*., 2004).

A toxidade do QCR em peixes foi estudada por diversos autores que demonstraram que, em nível subletal, os efeitos incluem a inibição da atividade AChE e dano oxidativo em tecidos *Leporinus obtusidens* (PRETTO *et al*., 2011), dano oxidativo em tecidos de *C. carpio* e alterações metabólicas em tecidos de *R. quelen* (TONI *et al*., 2013; MENEZES *et al*., 2014; PERSCH *et al*., 2017; PERSCH *et al*., 2018). Ainda, Fathy *et al*. (2019) observaram que o QCR foi capaz de induzir

alterações nos eritrócitos em peixes juvenis da espécie *Oreochromis niloticus* expostos a uma concentração subletal do herbicida de 11,25 µg/mL, por um período de 96 horas.

Tabela 3 – Ecotoxicidade em peixes e toxicidade aguda em ratos de laboratório do principal produto à base de quincloraque comercializado no Brasil, na formulação de pó molhável (WP)[(1)].

	Ecotoxicidade		**Efeitos agudos em ratos**		
Nome do produto (comercial)	**CL_{50}[(3)](96h) *Salmo gairdneri*** (mg/L)	**CL_{50}(96h) *Poecilia Reticulata*** (mg/L)	**DL_{50}[(4)]oral** (mg/kg p.c.)	**DL_{50} dérmica** (mg/kg p.c.)	**CL_{50} (4h) inalatória** (mg/L)
FACET® (50% m/m)[(2)]	> 100	307,34	4120	> 2000	> 5,15

[(1)]Water Power; [(2)]Ingrediente ativo; [(3)]Concentração Letal Média capaz de causar a mortalidade da metade dos organismos; [(4)]Dose Letal Média capaz de causar a mortalidade da metade dos organismos. Fonte: MAPA/AGROFIT, 2024; FISPQ: BASF/2021 – Internet.

3.2 Testes de estabilidade térmica dos agroquímicos

A combustão indesejada dos agroquímicos pode ocorrer durante incidentes, acidentes ou acidentes graves. A degradação térmica e a combustão dessas substâncias podem levar à emissão de vários produtos tóxicos que podem ser nocivos aos seres humanos e ao meio ambiente (BORUCKA; CELIŃSKI, 2019). Além disso, segundo Senneca; Scherillo; Nunziata, (2007), diversos fatores podem favorecer a decomposição térmica e a combustão dos agroquímicos, dentre eles, o armazenamento inadequado de grandes quantidades desses produtos. Portanto, determinar o comportamento de inflamabilidade e conhecer quais os produtos de degradação térmica resultantes pode contribuir para uma análise e gerenciamento de risco dos processos de produção, armazenamento e distribuição desses produtos químicos (BORUCKA; CELIŃSKI, 2019).

Algumas metodologias, como as análises térmicas, auxiliam para determinar os produtos tóxicos liberados na decomposição térmica de agroquímicos, por exemplo. Estas empregam um grupo de técnicas analíticas nas quais uma propriedade física ou química de uma substância, ou de seus produtos de reação, é monitorada em função do tempo ou temperatura, enquanto a temperatura da

amostra, sob uma atmosfera específica, é submetida a uma programação controlada (CHEN *et al*., 2012). Normalmente, a caracterização térmica dos compostos químicos é realizada utilizando a análise termogravimétrica (TGA) e a análise de calorimetria exploratória diferencial (DSC), em laboratório. A determinação da estabilidade térmica dos compostos pode ser realizada a partir da TGA, enquanto que a DSC mede a diferença na quantidade de calor necessária para aumentar a temperatura de uma amostra de teste, em relação ao material de referência em função da temperatura (CHEN *et al*., 2012; FRIZZO *et al*., 2013).

3.3 Linhagem celular hepática *Zebrafish-Liver* (ZF-L)

A espécie *D. rerio* (família: Cyprinidae; ordem: Cypriniformes) é um pequeno peixe ósseo, nativo dos rios do sul e sudeste da Ásia, que possui boa adaptação a vida em aquário. Quando adulto, pode chegar a medir de 3 a 5 cm (BRAUNBECK; LAMMER, 2006; CANEDO; ROCHA, 2021). Este peixe é conhecido popularmente no mundo e na comunidade científica como *zebrafish*, na qual passou a ter relevância no início da década 1980, a partir de publicações utilizando o modelo no estudo do desenvolvimento e função do sistema nervoso, realizadas pela equipe do biólogo norte-americano George Streisinger, cujos trabalhos começaram a ser publicados a partir de 1981 (GRUNWALD; EISEN, 2002; KÜTTER *et al*., 2023). No Brasil, é também conhecido como paulistinha ou peixe-zebra.

O peixe-zebra foi a primeira espécie de peixe a ter o genoma totalmente seqüenciado, possuindo pelo menos 70% de genes homólogos entre este animal e humanos (HOWE *et al*., 2013; TRIGUEIRO *et al*., 2020). Dadas às semelhanças fisiológicas, morfológicas e histológicas com os mamíferos, o torna um organismo modelo de grande interesse em diversas linhas de pesquisa, incluindo os estudos toxicológicos (DE SOUZA ANSELMO *et al*., 2018; CASSAR *et al*., 2020; GONÇALVES *et al*., 2020; CUI *et al*., 2023). Por esta razão, modelos de peixe-zebra têm sido usados para investigar diversos tipos de doenças e distúrbios humanos e, devido a sua grande sensibilidade quando expostos a produtos químicos ao absorver de forma rápida os compostos que são diretamente adicionados na água, também podem ser utilizados em pesquisas de medicamentos candidatos para essas doenças (GOESSLING; SADLER, 2015; WILKINS; PACK, 2016; TRIGUEIRO *et al*., 2020; KATOCH; PATIAL, 2021).

Além disso, o peixe-zebra também é um organismo já bem estabelecido como bioindicador em estudos toxicológicos de poluentes ambientais, tanto que é um modelo utilizado em muitas diretrizes ecotoxicológicas padrão da OECD (*Organization for Economic Co-Operation and Development*), sendo amplamente utilizado em testes de laboratório (BRAUNBECK; LAMMER, 2006; BUSQUET *et al*., 2014; VON HELLFELD *et al*., 2020; CANEDO; ROCHA, 2021). Portanto, este peixe é de grande interesse para ensaios que visam ao monitoramento de sistemas aquáticos que podem ter sido contaminados por toxinas ambientais prejudiciais à saúde, como por exemplo, agroquímicos (BAMBINO; CHU, 2017; LI *et al*., 2020; GONÇALVES *et al*., 2020).

No entanto, é importante ressaltar que o uso de animais vertebrados em pesquisas, envolve questões éticas e ao bem-estar animal. Além disso, muitas vezes, requerem um número considerável de animais, são demorados e produzem maior volume de resíduos contaminados (TAJU *et al*., 2017; KATAGI, 2020; EMBERLEY-KORKMAZ *et al*., 2023). Desta forma, surgem as propostas de uso de modelos alternativos dentro do princípio dos 3Rs (*Replacement*, *Reducion and Refinement* – Substituição, Redução e Refinamento), nos quais também podem ser incluídos os matemáticos, *in sílico*, testes de fototoxicidade, bem como os ensaios *in vitro* utilizando linhagens celulares de animais (ARAÚJO *et al*., 2014; FISCHER; MILTON; WALLACE, 2020; ALEHASHEM *et al*., 2022). Neste sentido, Alehashem *et al*. (2022) enfatizam que, embora a utilização de dados *in vitro* para avaliação de riscos ainda representa um desafio devido às limitações, ensaios *in vitro* utilizando células podem contribuir para a caracterização dos perigos e identificar os mecanismos envolvidos na toxicidade de agroquímicos, por exemplo.

O modelo *in vitro* de cultura celular, consiste no isolamento, manutenção asséptica e propagação de células, órgãos ou tecidos fora do organismo, em um ambiente artificial nas condições nutricionais e ambientais favoráveis, aplicável a culturas de células primárias ou linhagens celulares estabelecidas (SCHIRMER, 2006; AVALOS-SORIANO; GARCÍA-GASCA; YAÑEZ-RIVERA, 2021). Portanto, em testes toxicológicos, experimentos utilizando linhagens celulares de vertebrados podem reduzir o uso de animais, apesar de não substituí-los, e também reduzem a quantidade de resíduos tóxicos gerados (BOLS *et al*., 2005; EMBERLEY-KORKMAZ *et al*., 2023).

Neste contexto, no que diz respeito ao cultivo celular de vertebrados e, considerando que o fígado é o órgão alvo para a maioria das toxinas (GOULART; BOYLE; SOUZA, 2015; LUNGU-MITEA *et al*., 2018), a linhagem celular estabelecida ZF-L derivada de tecido hepático normais do peixe-zebra (*D. rerio*) adulto (MIRANDA *et al*., 1993; GHOSH; ZHOU; COLLODI, 1994) tem sido utilizada para avaliações de toxicidade e de estresse oxidativo de várias substâncias, como por exemplo, nanomateriais e metais (SANDRINI *et al*., 2009; COSTA *et al*., 2012; MOROZESK *et al*., 2018; KWOK; CHAN, 2020; MOROZESK *et al*., 2020; FLEURBAIX *et al*., 2022), biodiesel e gasolina (CAVALCANTE *et al*., 2014; LACHNER; OLIVEIRA; MARTINEZ, 2015) e agroquímicos (GOULART; BOYLE; SOUZA, 2015; BONOMO *et al*., 2019; LOPES; SANDRINI; SOUZA, 2018; BONOMO *et al*., 2020).

Em relação a estudos de toxicidade de agroquímicos utilizando células ZF-L, Bonomo *et al*. (2019) demonstraram que o inseticida metálico hesperidina (MgHP), após 24 horas de exposição, reduziu a atividade mitocondrial nas concentrações de 0,001 a 1 µg/mL, enquanto a integridade lisossomal, avaliada pelo ensaio do vermelho neutro, diminuiu em todas as concentrações testadas (0,0001 a 1 µg/mL) Ainda em relação à avaliação da viabilidade celular, An *et al*. (2024) avaliaram os efeitos citotóxicos do inseticida piridabeno nas células ZFL utilizando o ensaio MTT e verificaram que o inseticida reduziu a viabilidade celular nas concentrações de 0,05 µg/mL e 0,2 µg/mL, após 48 horas de exposição.

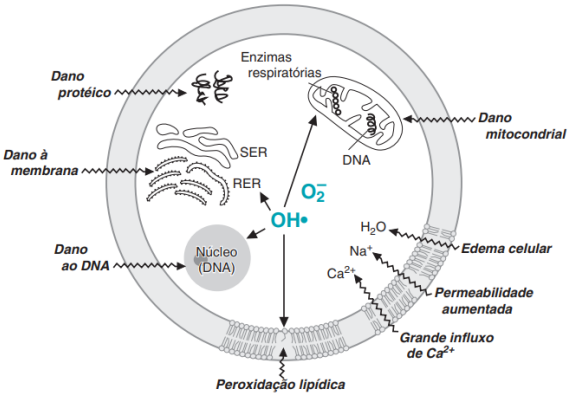
Com relação à toxicidade de herbicidas em células ZF-L, Goulart; Boyle; Souza (2015) investigaram a citotoxicidade do herbicida de Roundup Transorb® (um herbicida à base de glifosato), em três alvos celulares (membrana celular, lisossomos e mitocôndrias) após 6 horas de exposição, utilizando os ensaios azul de tripan, vermelho neutro e MTT, respectivamente. Foi observada uma redução na membrana celular e na integridade lisossomal nas concentrações de 0,0677, 0,1354 e 0,2708 µg/mL e uma redução na atividade metabólica após exposição a 0,1354 e 0,2708 µg/mL. Também, Lopes; Sandrini; Souza, (2018), utilizando concentrações de 0,65 e 3,25 µg/mL de Roundup®, observaram uma redução na atividade mitocondrial e na integridade lisossomal nas células ZF-L, após 24 horas de exposição ao herbicida, na concentração de 3,25 µg/mL.

3.4 Estresse oxidativo

O estresse oxidativo (EO) é um processo fisiológico que ocorre nas células e tecidos quando há um desequilíbrio entre a produção de espécies reativas (ER) e a capacidade antioxidante em neutralizá-las. Dentre as ER, o oxigênio (O_2) pode dar origem às espécies reativas de oxigênio (ERO), que incluem radicais livres (RL) como o ânion superóxido ($O_2^{\bullet -}$) e o radical hidroxil ($^{\bullet}OH$) e espécies não-radicais, como o peróxido de hidrogênio (H_2O_2) (HALLIWELL; WHITEMAN, 2004; SULE; CONDON; GOMES, 2022). RL são átomos ou moléculas que apresentam um elétron desemparelhado em sua última camada eletrônica; em geral, são instáveis com grande aptidão para reagir com diversos compostos e estruturas celulares e, em se tratando de ERO, o elétron desemparelhado está localizado no átomo de oxigênio (HALLIWELL, 2007; COSTANTINI, 2014; DI MEO; VENDITTI, 2020).

A geração de ERO é um processo que ocorre normalmente nas mitocôndrias, nas membranas celulares e no citoplasma, cumprindo funções biológicas essenciais para a manutenção fisiológica dos organismos de modo saudável (COSTANTINI, 2014; DI MEO; VENDITTI, 2020). No entanto, quando são produzidas em excesso, as ERO acabam se tornando tóxicas às funções vitais, promovendo alterações no estado redox, podendo causar danos oxidativos a macromoléculas como o DNA (ácido desoxirribonucleico), interagindo com o material genético e favorecer a genotoxicidade. Também podem causar oxidação às proteínas, oxidar biomoléculas como os lipídios, levando à peroxidação lipídica das membranas celulares (Figura 3) e, por fim, podem levar à morte celular, seja por necrose ou apoptose (JABŁOŃSKA-TRYPUĆ, 2017; LUSHCHAK *et al*., 2018).

Figura 3 – Danos induzidos por radicais livres na célula.



Fonte: SMITH; MARKS; LIEBERMAN, 2007.

Através da reação de Haber-Weiss (Figura 4), o ânion superóxido ($O2^{\bullet-}$) ao reagir com o H_2O_2 pode dar origem ao radical hidroxil ($^{\bullet}OH$), sendo este mais danoso à célula (COSTANTINI, 2014).

Figura 4 – Reação do ânion superóxido com o peróxido de hidrogênio resultando em radical hidroxil.

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{-}OH + {}^{\cdot}OH$$

Fonte: COSTANTINI, 2014.

Com o objetivo de evitar danos oxidativos causados pelo excesso de produção de ERO, as células possuem vários mecanismos de defesa antioxidantes, com grande potencial para combater as ações das ER (LUSHCHAK *et al*., 2018). Desta forma, o sistema antioxidante atua na proteção das células contra os danos das ERO, neutralizando os efeitos do EO. Este sistema é classificado em não enzimáticos, por exemplo, a glutationa (GSH) e enzimáticos, incluindo a SOD e a CAT (JABŁOŃSKA-TRYPUĆ, 2017; LUSHCHAK *et al*., 2018).

Conforme demonstrado na reação apresentada na Figura 5, em reação simultânea, a SOD aparece como sendo a primeira enzima antioxidante a atuar na redução do $O_2^{\bullet-}$, um radical altamente reativo, em H_2O_2, um oxidante fraco. Na sequência, a enzima CAT transforma o H_2O_2 em H_2O e O_2 (Figura 5) (COSTANTINI, 2014). Portanto, quando ocorre um aumento da atividade da SOD, esta deve ser acompanhada pelo aumento da enzima CAT, para evitar o acúmulo do H_2O_2 e, assim, evitar a ocorrência de danos às celulas (SULE; CONDON; GOMES, 2022).

Figura 5 – Reações de transformação de espécies reativas pelas enzimas SOD e CAT em moléculas não reativas.

$$2O_2^{\cdot-} + 2H^{+} \xrightarrow{SOD} O_2 + H_2O_2$$
$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Fonte: COSTANTINI, 2014.

A indução de estresse oxidativo por agroquímicos em diferentes tipos de linhagens celulares, células e tecidos de animais, incluindo peixes, está bem

relatada na literatura (SLANINOVA *et al*., 2009; DEYASHI; CHAKRABORTY, 2016; SULE; CONDON; GOMES, 2022).

Em relação ao herbicida IMZT, Moraes *et al*. (2011) relataram aumento atividade da CAT em tecido hepático do peixe *C. carpio* após exposição de 7 dias à uma mistura comercial contendo IMZT e imazapic (0,0985 e 0,0209 µg/mL, respectivamente). Yao *et al*. (2020) observaram que camundongos tratados com 100 mg/Kg por 16 dias com enantiômero S(-) IMZT ou R(+) IMZT, o S(-) IMZT levou a um aumento da atividade da CAT no fígado, em comparação R(+) IMZT.

Em relação a peixes expostos ao herbicida QCR, Pretto *et al*. (2011) mostraram a atividade da CAT hepática reduzida do peixe *L. obtusidens*, após 90 dias exposição à formulação comercial contendo QCR, a uma concentração de 0,204 µg/mL. Toni *et al*. (2013) observaram uma diminuição das atividades de CAT no fígado do peixe *C. carpio,* após a exposição a uma formulação comercial contendo QCR na concentração de 0,3446 µg/mL após 30 dias. Menezes *et al*. (2012) mostraram que o QCR diminuiu a atividade de SOD no fígado do peixe *C. carpio* expostos a 1 µg/mL, por 8 dias. O mesmo foi observado em peixes *R. quelen* (MENEZES *et al*., 2014). Além disso, também foram relatados aumentos das atividades das enzimas SOD e CAT no fígado de peixes juvenis da espécie *O. niloticus* expostos ao QCR na concentração de 11,25 µg/mL, por um período de 96 horas (SALEH *et al*., 2022).

4 Resultados

Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo e um manuscrito. As seções materiais e métodos, resultados, discussão e referências encontram-se nos próprios artigo e manuscrito.

O artigo está estruturado de acordo com a revista em que foi publicado e o manuscrito, da mesma forma, estruturado conforme a revista à qual foi submetido.

4.1 Capítulo 1 – Artigo

Effects of imazethapyr-based herbicide formulation in the zebrafish (*Danio rerio*) hepatocyte cell line (ZF-L): cytotoxicity and oxidative stress

Edila Maria Kickhöfel Ferrer, Milena Mattes Cerveira, Pedro Henrique Flores da Cruz, Cleiton Jesus Andrade Pereira, Lucas Moraes Berneira, Claudio Martin Pereira de Pereira, Nathalia Stark Pedra, Roselia Maria Spanevello, Jean Carlos Bauer Vieira, Clarissa Piccinin Frizzo, Mateus Tavares Kütter, Janice Luehring Giongo, Rodrigo de Almeida Vaucher

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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

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(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.

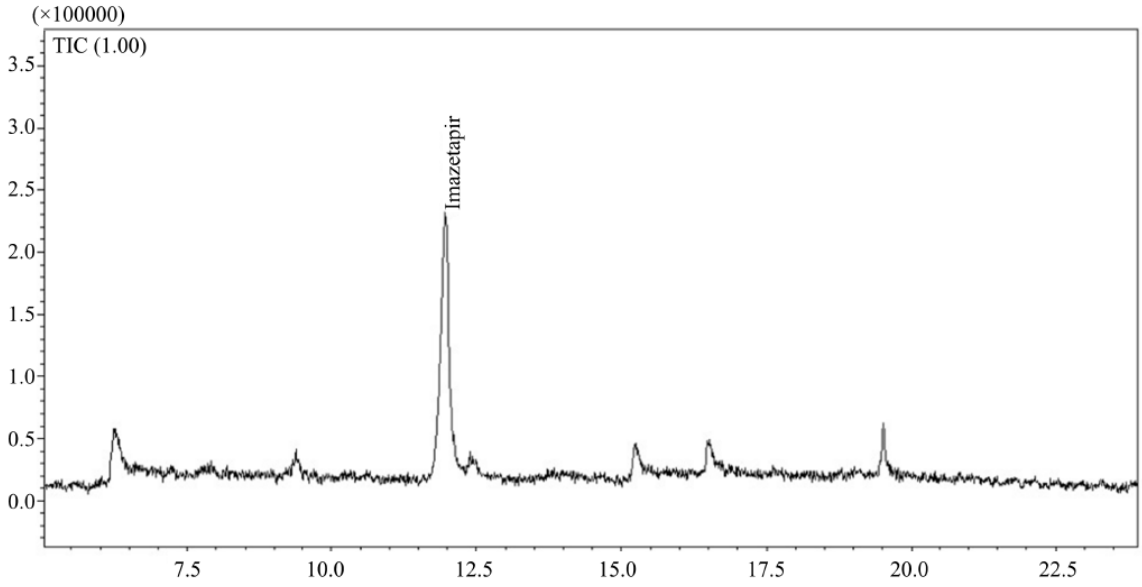


Figure 1. Representative chromatogram of IMZT.

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.

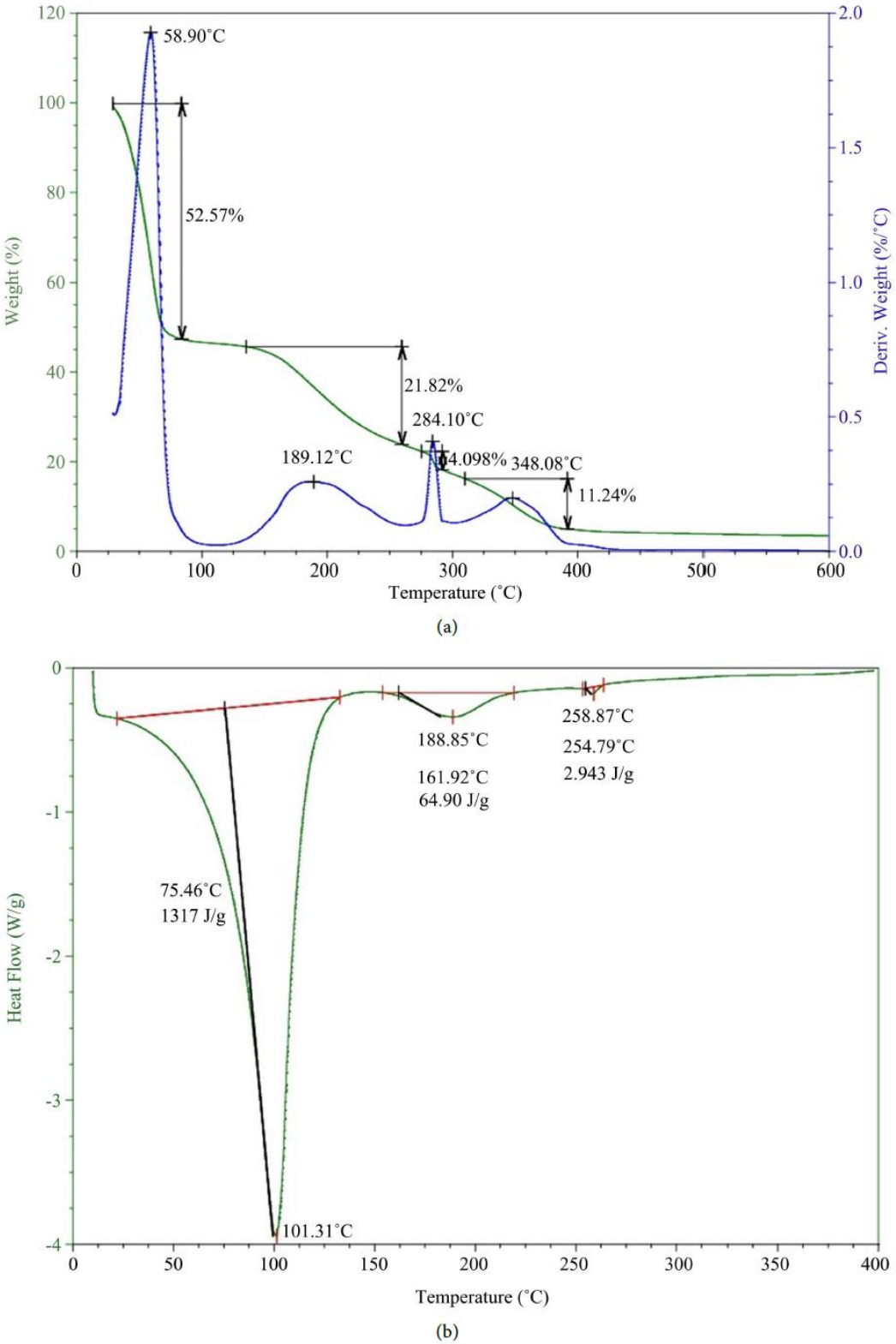


Figure 2. Thermal response curves for the IMZT herbicide formulation. (a) TGA and DTG curve; (b) DSC curve.

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.

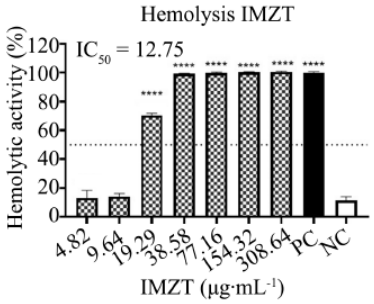


Figure 3. Hemolytic activity of IMZT in animal erythrocytes. Data is expressed as mean ± SD, whereas the mean of positive control was used as 100%. Triton X-100 and saline were used as positive and negative controls, respectively. Data were analyzed using a one-way ANOVA followed by Dunnett's post-hoc 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

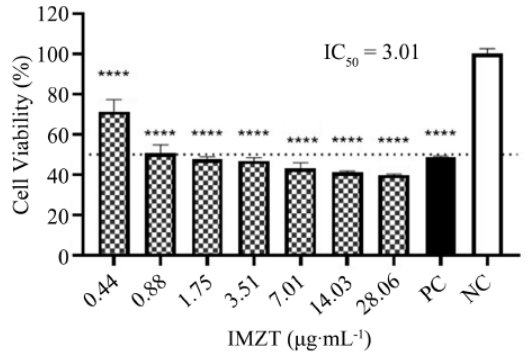
Evaluation of cytotoxic activity on mitochondria was performed using the MTT assay. The values are presented in Figure 4(a). ZF-L cells exposed to IMZT exhibited reduced mitochondrial activity after 24 h of exposure at all concentrations used (0.44 - 28.06 µg/mL) in 28.52%, 49.45%, 52.33%, 53.03%, 56.78%, 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

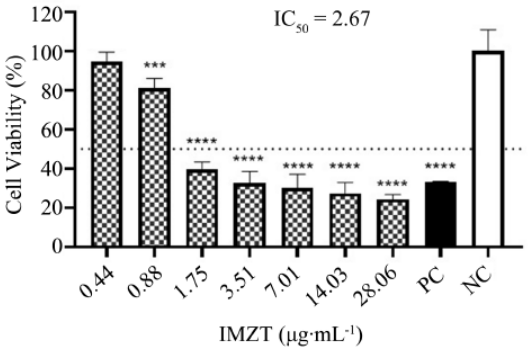
Results of the evaluation of cytotoxic activity on lysosomes are expressed in Figure 4(b). Treatment with IMZT reduced the lysosomal activity of ZF-L cells after 24 h of exposure at concentrations of 0.88 to 28.06 µg/mL in 18.77%, 60.45%, 67.29%, 69.80%, 72.56%, and 75.66% respectively, when compared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

3.3.4. LDH Release Assay

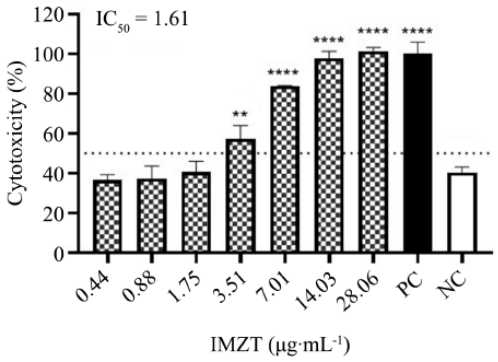
LDH release assay was performed using the supernatant medium of cells exposed to IMZT for 24 hours. It was observed that the herbicide affected the membrane integrity, and cellular leakage a significant increase at concentrations of 3.51 to 28.06 µg/mL in 57.36%, 83.81%, 97.81%, and 101.31% respectively. 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)



(b)



(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.

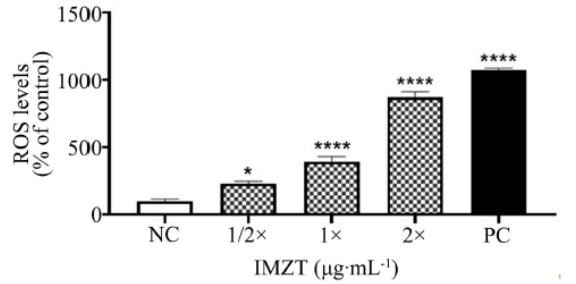
3.3.6. Oxidative Stress Effects on ZF-L Cells

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-

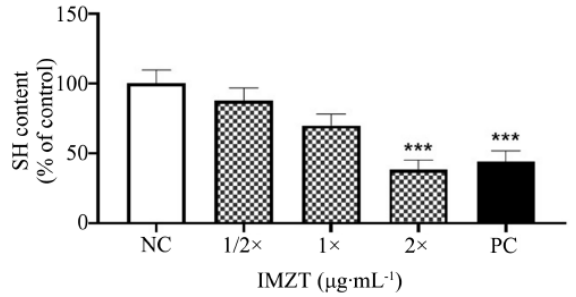
responding to the median IC_{50} values in Table 1. The concentrations used were: 1/2× (1.42 μg/mL), 1× (2.84 μg/mL), and 2× (5.68 μg/mL).

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.

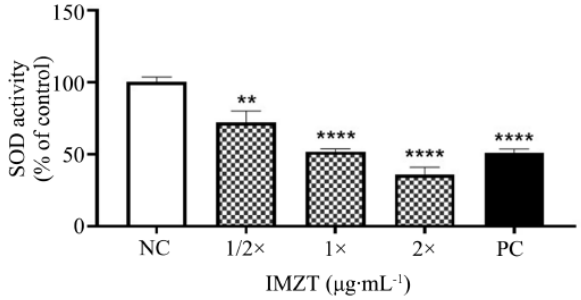
Interestingly, regarding the enzymatic activity of antioxidants (SOD and CAT), it was noted that IMZT reduced the activity of these enzymes at all tested concentrations compared to NC (Figure 5(c) and Figure 5(d)). Specifically, SOD activity exhibited reductions of 28.11%, 48.32%, and 64.11%, while CAT 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).



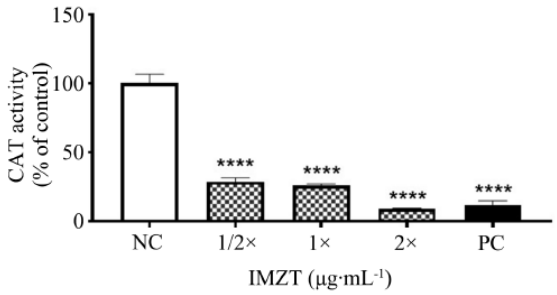
(a)



(b)



(c)



(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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4.2 Capítulo 2 – Manuscrito

Assessment of cytotoxicity and oxidative stress in the zebrafish liver cell line (ZF-L) exposed to a seized formulation of the herbicide quinclorac (QCR).

Avaliação da citotoxicidade e do estresse oxidativo na linhagem celular de fígado do *zebrafish* (ZF-L) exposta a uma formulação apreendida do herbicida quinclorac (QCR).

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Abstract

The seizure of agrochemicals is a persistent issue in Brazil, particularly in states bordering other South American countries, which experience the highest levels of agrochemical confiscations. We evaluated biochemical parameters including oxidative stress, hepatotoxicity, and cytotoxicity of the herbicide quinclorac (QCR) using a sample obtained from a seizure. The herbicide was characterized using Gas Chromatography-Mass Spectrometry and thermal analysis. Additionally, hemolytic activity, along with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), neutral red (NR), and lactate dehydrogenase (LDH) assays in Zebrafish-Liver (ZF-L) cells, were performed to assess QCR cytotoxicity. The results showed IC_{50} values of 19.83 µg/mL for erythrocytes and 1.78 µg/mL, 5.13 µg/mL, and 0.95 µg/mL for MTT, NR, and LDH assays, respectively. A median IC_{50} of 3.46 µg/mL was used for cholinesterase (COL) and aspartate aminotransferase (AST) dosages, as well as oxidative stress assays, which revealed an increase in reactive oxygen species (ROS) production, along with a decrease in total thiol (SH) content and the activities of superoxide dismutase (SOD) and catalase (CAT). Furthermore, QCR exposure resulted in elevated AST levels, indicative of liver damage, and a biphasic response in COL activity, with a significant reduction at higher concentrations. Our research indicates that QCR induces hepatotoxicity and cytotoxicity via oxidative stress in the ZF-L cell line under *in vitro* conditions.

Keywords: agrochemical; quinolinecarboxylic acid; cell viability; hepatocytes.

Resumo

A apreensão de agroquímicos é um problema persistente no Brasil, particularmente em estados que fazem fronteira com outros países da América do Sul, que apresentam os maiores níveis de confiscos de agroquímicos. Avaliamos parâmetros bioquímicos, incluindo estresse oxidativo, hepatotoxicidade e citotoxicidade do herbicida quinclorac (QCR) usando uma amostra obtida de uma apreensão. O herbicida foi caracterizado usando cromatografia gasosa-espectrometria de massas e análise térmica. Além disso, a atividade hemolítica, juntamente com ensaios de MTT (brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio), vermelho neutro (NR) e lactato desidrogenase (LDH) em células de fígado de peixe-zebra (ZF-L), foram realizados para avaliar a citotoxicidade do QCR. Os resultados mostraram valores de IC_{50} de 19,83 µg/mL para eritrócitos e 1,78 µg/mL, 5,13 µg/mL e 0,95

µg/mL para ensaios de MTT, NR e LDH, respectivamente. Uma mediana de IC_{50} de 3,46 µg/mL foi usada para dosagens de colinesterase (COL) e aspartato aminotransferase (AST), bem como ensaios de estresse oxidativo, que revelaram um aumento na produção de espécies reativas de oxigênio (ROS), juntamente com uma diminuição no conteúdo total de tiol (SH) e nas atividades da superóxido dismutase (SOD) e catalase (CAT). Além disso, a exposição ao QCR resultou em níveis elevados de AST, indicativos de dano hepático, e uma resposta bifásica na atividade do COL, com uma redução significativa em concentrações mais altas. Nossa pesquisa indica que o QCR induz hepatotoxicidade e citotoxicidade por meio de estresse oxidativo na linhagem celular ZF-L em condições *in vitro*.

Palavras-chave: agroquímico; ácido quinolinocarboxílico; viabilidade celular; hepatócitos.

1. Introduction

Agrochemicals are intricate mixtures designed to eliminate or manage pests, thereby boosting agricultural output (Mesnage and Antoniou, 2017; Lushchak et al., 2018; Tudi et al., 2021). However, due to their toxic nature, these substances contribute to the pollution of not only food but also the soil, water, and atmosphere. Consequently, the application of these chemicals can have adverse effects on the health of non-target species (Tudi et al., 2021; Ziliotto et al., 2023). Globally, herbicides are the most widely used agrochemicals, followed by insecticides and fungicides (Sharma et al., 2020; Leoci and Ruberti, 2021).

QCR (3,7-dichloro-8-quinolinecarboxylic acid) is extensively used in the agricultural sector to control weeds in irrigated rice cultivation and to control glyphosate-resistant monocots (Coltro et al., 2017; Reichert et al., 2023). Although QCR is considered to have low toxicity for several non-target organisms, its toxicity in non-target vertebrate aquatic organisms such as amphibians and fish have been described (Pretto et al., 2011; Toni et al., 2013; Dornelles and Oliveira, 2014; Coltro et al., 2017; Persch et al., 2018; Fathy et al., 2019). In Brazil, there is a recurring issue related to the trafficking of agrochemicals, particularly in states that share borders with other Latin American countries, such as Rio Grande do Sul (Lemos et al., 2018). This results in the acquisition of agrochemicals with altered formulations, especially in these states. The issue becomes even more significant when the

smuggled agrochemicals are subject to strict regulations, as is the case with QCR, with some of these formulations being seized by federal authorities (Lemos et al., 2018; Berneira et al., 2020).

The toxicity of agrochemicals, including herbicides, has been widely studied in both *in vitro* and *in vivo* models (Gonçalves et al., 2020; Nagy et al., 2020). However, there is a lack of research focused on the *in vitro* toxicity assessment of seized agrochemical formulations. Therefore, the aim of this study was to evaluate the *in vitro* biological parameters related to the toxicity of a formulation containing the herbicide QCR, which was confiscated in Jaguarão, Rio Grande do Sul, Brazil, using hepatocytes of zebrafish (ZF-L). The cell line was chosen due to its demonstrated sensitivity to agrochemical exposure in toxicity assessments, as shown in multiple studies, and because it originates from an aquatic vertebrate species (*Danio rerio*) that is more susceptible to the toxic effects of agrochemicals (Goulart et al., 2015; Lopes et al., 2018; Bonomo et al., 2019, Bonomo et al., 2020). Cytotoxicity, oxidative stress tests and biomarker dosing were performed using the ZF-L cell line to assess the toxic effects of the seized formulation containing the herbicide QCR.

2. Materials and methods

2.1. Materials

Cell media (Leibovitz's L-15, Ham's F-12 and high glucose DMEM) were obtained from Vitrocell Embriolife (São Paulo, Brazil) and defibrinated sheep blood was obtained with Laborclin (Paraná, Brazil). All remaining reagents and solvents were of analytical or pharmaceutical quality. A formulation of QCR, seized by the Brazilian Federal Police (PF), was generously provided for use in this study (Berneira et al., 2020).

2.2. *Agrochemical extraction and identification*

For extraction, 90 mg of the agrochemical formulation was mixed with 25 mL of acetonitrile. Following a 5-minute centrifugation, 1 µL of the solvent fraction was injected into a GC-MS QP2010SE (Shimadzu®) operating in split mode (1:25). Helium was used as the carrier gas, and the capillary column employed was an Rtx-5MS model. The injector temperature was maintained at 260°C, and the ion source was set to 280°C. The column temperature program started at 40°C for 3 minutes, then increased at a rate of 10°C/min to 280°C, which was held for 10 minutes. Mass

spectrometry was conducted in scan mode over a range of 50 to 550 m/z (Berneira et al., 2020).

2.3. Thermogravimetric Analysis (TGA)

TGA of the QCR herbicide formulation was conducted using a TGA Q5000 (TA Instruments Inc., USA). The instrument was calibrated with $CaC_2O_4.H_2O$ (99.9%), and a heating rate of 10°C/min was applied under an inert nitrogen gas (N_2) atmosphere (25 mL/min). The sample mass was 5.7420 mg. Data analysis was carried out using TA Universal Analysis 2000 Software, version 4.5 (TA Instruments Inc., USA).

2.4. Differential scanning calorimetry (DSC)

DSC analysis of the QCR herbicide formulation was carried out on MDSC Q2000 (TA Instruments, USA) equipped with the MDSC option. The instrument included an RCS90 cooling accessory and used N_2 as purge gas at 50 mL/min. The heating rate was set to 5°C/min. Calibration of the instrument was done using indium (In, 99.99%) in the DSC standard form. The sample, with a mass of 5.140 mg (± 0.001 mg), was precisely weighed. Data were analyzed using TA Universal Analysis 2000 Software, version 4.5 (TA Instruments Inc., USA).

2.5. QCR formulation cytotoxicity

2.5.1. Hemolytic assay (HA)

For the hemolysis assay, commercial defibrinated sheep blood was used, with protocol modifications from Cerveira et al. (2021). Erythrocytes were washed with 0.9% (m/v) saline (NaCl) and centrifuged at 2500 rpm for 15 minutes. A 4% (v/v) suspension of erythrocytes was prepared and incubated with the herbicide QCR at concentrations of 2.63, 5.27, 10.54, 21.08, 42.16, and 84.82 µg/mL at 37 ºC for 1 hour. After incubation, the samples were centrifuged at 2500 rpm for 10 minutes. The supernatants were transferred to a 96-well plate, and absorbance was recorded at 419 nm. Saline solution and Triton-X 100 (0.1%) were used as negative and positive controls, respectively. The hemolysis rate (%) was obtained from the equation $[(A_S - A_N) / (A_S - A_P)] \times 100$, where A_S is the absorbance of the treated sample, A_N is the absorbance of the negative control, and A_P is the absorbance of the positive control.

2.5.2. Zebrafish-Liver (ZF-L) cell line culture

The hepatocites ZF-L was obtained from the Rio de Janeiro Cell Bank (BCRJ–Brazil). Cells were cultured in flasks and maintained in a complete medium (50% L-15, 35% high-glucose DMEM, 15% Ham's F-12). The medium was supplemented with buffering agents (0.15 g/L NaHCO3, 15 mM HEPES), 0.01 mg/mL insulin, heat-inactivated FBS and antibiotics (50 µg/mL enrofloxacin) and antimicotics (2.5 µg/mL amphotericin B) as recommended by the cell bank. Cultured cells were kept at 28ºC in an incubator.

2.5.3. QCR treatment of cell culture

To evaluate cytotoxicity, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and neutral red (NR) uptake assay were used, along with the lactate dehydrogenase (LDH) release assay as an indirect marker of cell lysis. Additionally, oxidative stress was evaluated using assays for superoxide dismutase (SOD), catalase (CAT), and total sulfhydryl groups (SH). ZF-L cells were seeded in 96-well plates at 3×10^4 cells per well and in 6-well plates at 6×10^5 cells per well. The plates were incubated for 48 hours at 28°C in a dry oven. The QCR herbicide samples were diluted in complete medium without FBS. For the MTT, NR, and LDH assays, concentrations tested were 0.24, 0.48, 0.96, 1.92, 3.83, and 7.67 µg/mL. To evaluate the oxidative stress in the cell line, concentrations of 1/2x, 1x, and 2x, based on the median IC_{50} from the cytotoxicity assays were used. After treatment, cells were incubated for 24 hours. In the LDH assay, Triton X-100 at 5% was employed as the positive control, while hydrogen peroxide (H_2O_2) at 0.5% (v/v) was used as the positive control for the MTT, NR assays, and oxidative stress assessments. For all assays, cells exposed exclusively to the culture medium acted as the negative control.

2.5.4. Mitochondrial viability assay

Mitochondrial viability was evaluated through the MTT assay, following the protocol established by Mosmann (1983). This technique depends on the mitochondria's capacity to reduce MTT (yellow), to formazan crystals (purple). After treating the cells with the specified concentrations, the culture supernatants were discarded, and the cells were rinsed with PBS. A 50 µL aliquot of 1 mg/mL MTT solution was then added to each well and incubated at 28°C for 3 hours. Following

incubation, the MTT solution was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance at 540 nm was measured using a SpectraMax M3 microplate reader. Cell viability was determined by the formula $(A_S/A_C) \times 100$, where A_S denotes the absorbance of treated cells and A_C denotes the absorbance of control cells.

2.5.5. Lysosomal integrity assay

Lysosomal integrity was evaluated using the neutral red (NR) uptake assay, adapted from Borenfreund and Puerner (1985). After incubating the cells with the test concentrations, they were rinsed with PBS, and 200 µL of neutral red solution (40 µg/mL) was added to each well. The cells were then incubated at 28°C for 3 hours. Post-incubation, the cells were washed with PBS and subsequently exposed to 150 µL of an acid-alcohol solution to release the dye. The absorbance at 540 nm was recorded using a SpectraMax M3 microplate reader. The lysosomal viability was determined by the same formula of the MTT assay.

2.5.6. LDH release assay

The LDH release assay, adapted from Vaucher et al. (2010), was used to measure membrane damage in ZF-L cells induced by QCR. Following incubation at the specified concentrations, supernatants were collected, and LDH release was quantified using an enzymatic kinetics assay with a commercial LDH (UV) kit (Bioclin®). An automated analyzer (Cobas MIRA®) was used to measure absorbance at 340 nm, following the manufacturer's instructions. LDH release (%) was calculated as $[(A_S - A_N) / (A_S - A_P)] \times 100$, same as the hemolysis rate.

2.6. Biomarker dosages

The biomarker dosages were adapted from Vaucher et al. (2010), and were used to measure enzyme activity in ZF-L cells induced by QCR. Total cholinesterase (COL) and aspartate aminotransferase (AST) acitivities were measured in the supernatants obtained from treated cells and used as biomarkers of hepatotoxicity. Untreated cells were used as negative control and to measure baseline COL and AST activity in this assay.

2.6.1. Total COL activity

COL activity was measured using an enzymatic kinetics assay with a commercial cholinesterase kit from Bioclin®. Absorbance at 405 nm was measured with Cobas MIRA® according to the instructions. COL activity was expressed as units of enzyme per liter (U/L).

2.6.2. AST activity

AST activity was measured using an enzymatic kinetics assay with a commercial AST kit (Bioclin®). Following the instructions, AST levels were measured at 405 nm also using a Cobas MIRA® analyzer. AST activity was expressed as units of enzyme per liter (U/L).

2.7. Parameters of oxidative stress

2.7.1. Determination ROS generation

Using the Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay, the amount of intracellular ROS generated in intact cells was measured (Ali et al., 1992). In summary, following a 24-hour QCR treatment at 1/2x (1.73 µg/mL), 1x (3.46 µg/mL), and 2x (6.92 µg/mL) concentrations, which correspond to the median IC_{50} value, cells were treated with 1 µM DCFH-DA for 30 minutes in 96-well plates at a density of $3x10^4$ cells/well. With excitation/emission at 488/525 nm, fluorescence was measured in a SpectraMax M3 microplate reader. The percentage of control (NC) was used to express the ROS production.

2.7.2. Preparation of lysates

Following a 24-hour incubation period, cells were treated with QCR at concentrations of 1/2x (1.73 µg/mL), 1x (3.46 µg/mL), and 2x (6.92 µg/mL) that corresponded to the median IC_{50} value. Lysates were generated using a scraper, which is a mechanical force, and centrifuged for 10 minutes at 4ºC at 1000 rpm. The supernatants were used to assess the oxidative parameters (SH, SOD, and CAT). Bovine serum albumin was used as a reference for measuring protein levels (Lowry et al., 1951).

2.7.3. Total SH content quantification

The SH content in cell lysates was assessed in accordance with Markesbery and Aksenov (2001). The reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols is the basis for this assay. This reaction produces a yellow derivative (TNB) in the form of an oxidized disulfide. The inclusion of DTNB started the reaction. Using a microplate reader SpectraMax M3, absorbance was measured at 412 nm, and the results were represented as a percentage of NC.

2.7.4. SOD activity

Cell lysate SOD activity was determined using the methodology outlined by Misra and Frdovich (1972). The foundation of this test is the suppression of adrenaline's auto-oxidation to adrenochrome. Using a microplate reader SpectraMax M3, absorbance was measured at 480 nm, and the results were represented as a percentage of the NC.

2.7.5. CAT activity

Cell lysate CAT activity was measured using the methodology outlined by Aebi (1984). The breakdown of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) serves as the basis for this procedure. For 180 seconds at 37ºC, the reaction was constantly observed at 240 nm. The findings were reported as a percentage of NC and were read using a microplate reader SpectraMax M3.

2.8. Statistical Analysis

Statistical Analysis for the HA, MTT, NR, and LDH assays and for COL and AST dosages was conducted using GraphPad Prism 8.0. Results were presented as the mean ± standard deviation from triplicate experiments. Statistical significance was assessed using one-way ANOVA, with subsequent Dunnett's post-hoc test for the HA, MTT, NR, and LDH assays, and Tukey's post-hoc test for oxidative stress parameters. IC_{50} values were calculated using non-linear regression analysis. A significance level of $p < 0.05$ was used to determine statistically significant differences between means.

3. Results

3.1. Herbicide identification

QCR was identified by GC-MS and the herbicide peak obtained as shown in the chromatogram (Figure 1) was confirmed by mass spectra (data not shown). The remaining peaks correspond to the components of the herbicide formulation.

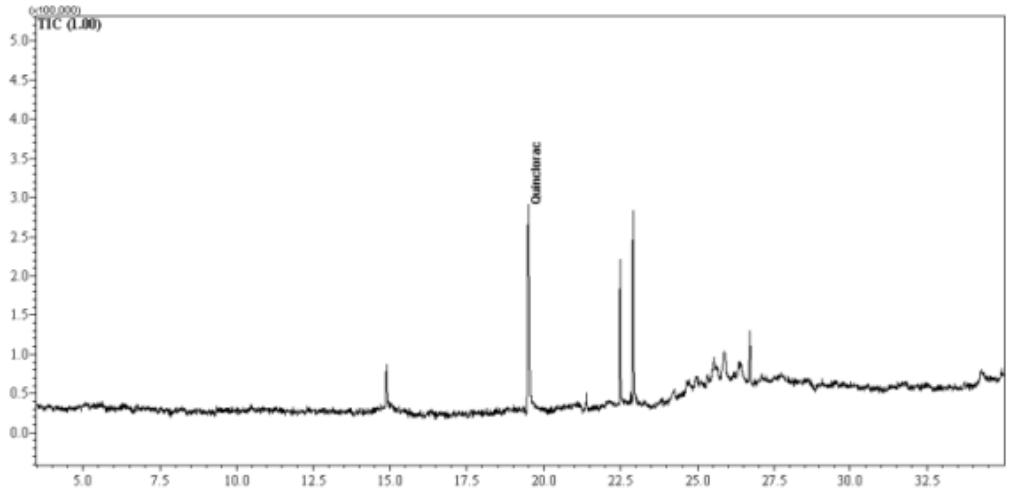

Figure 1. Representative QCR chromatogram

3.2. TGA and DSC

Figure 2 presents the results of the TGA and derivative (DTG) curves and the DSC curve of the herbicide QCR formulation. In the TGA and DTG analysis, peaks represent thermal events observed in the TGA curve, which are confirmed by the DTG curve showing mass loss relative to temperature.

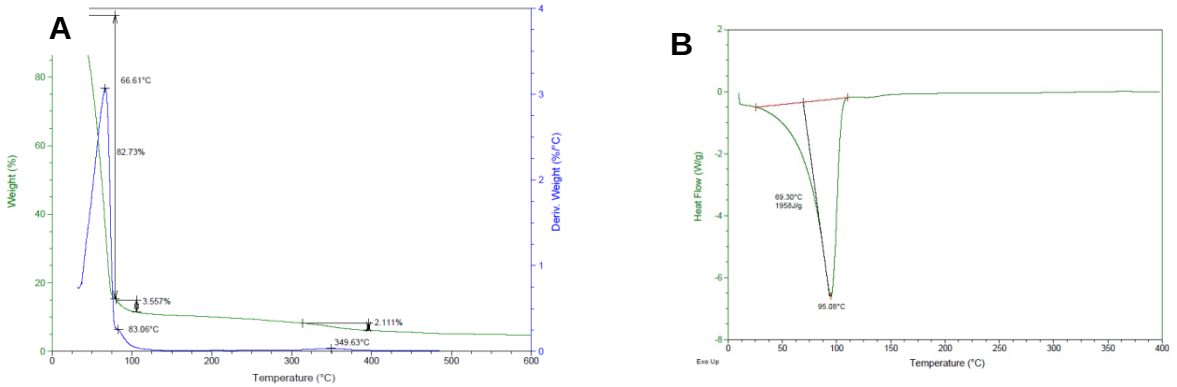

Figure 2. Representative thermal response curves for the QCR: (A) TGA and DTG and (B) DSC curves

As shown in Figure 2-A, the sample exhibited three distinct events. The first event involved a mass loss of 82.73% at a midpoint temperature of 66.61°C. The second event showed a 3.56% mass loss at a midpoint of 83.06°C. The third event displayed a decrease of approximately 2% at a midpoint of 350°C. No further mass changes were observed beyond 400°C. Figure 2-B presents the DSC results for the herbicide, illustrating the physical-chemical transition of the sample through energy variation with temperature. The sample exhibited a single event with an enthalpy change of 1958 J/g at 69.30°C, with a midpoint temperature of 95.08°C.

3.3. Cytotoxicity Assays

3.3.1. Hemolysis assay

Figure 3 shows the effects of the QCR herbicide formulation on hemolysis (HA), measured as the percentage of lysis in defibrinated sheep blood erythrocytes. The results reveal that QCR induced complete hemolysis (100%) at the highest concentrations of 42.16 and 84.32 µg/mL, and 51.58% hemolysis at 21.08 µg/mL, compared to the control. The IC_{50} for this assay was 19.83 µg/mL.

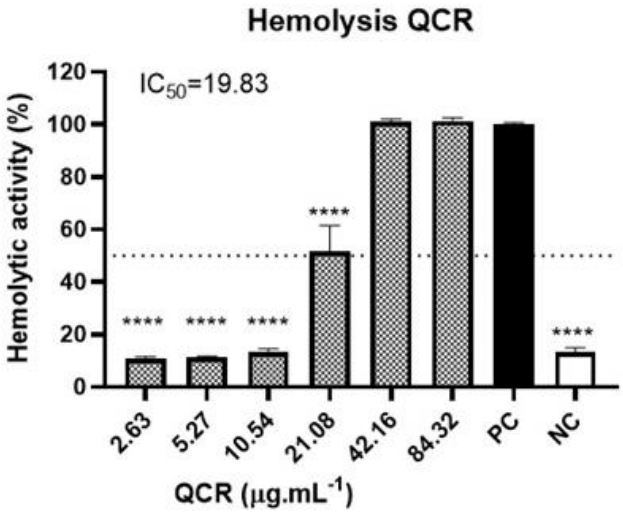

Figure 3. Hemolytic activity of QCR in sheep erythrocytes. Data is expressed as mean ± SD, whereas mean of positive control (PC) was used as 100%. Triton X-100 and saline were used as positive and negative controls, respectively. Data were analyzed using a one-way ANOVA followed by Dunnett's post-hoc test to determine the significance level where ****p <0.0001, in comparison to the 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. Three independent experiments were carried out in triplicate.

3.3.2. Mitochondrial viability

Mitochondrial cytotoxicity was assessed using the MTT assay, with results shown in Figure 4-A. ZF-L cells exposed to the QCR herbicide formulation demonstrated decreased mitochondrial activity after 24 hours at concentrations of 0.48 to 7.67 µg/mL, showing reductions of 36.69%, 43.36%, 65.01%, 69.72%, and 69.83%, respectively, compared to the control. The IC_{50} value for this assay was 1.78 µg/mL.

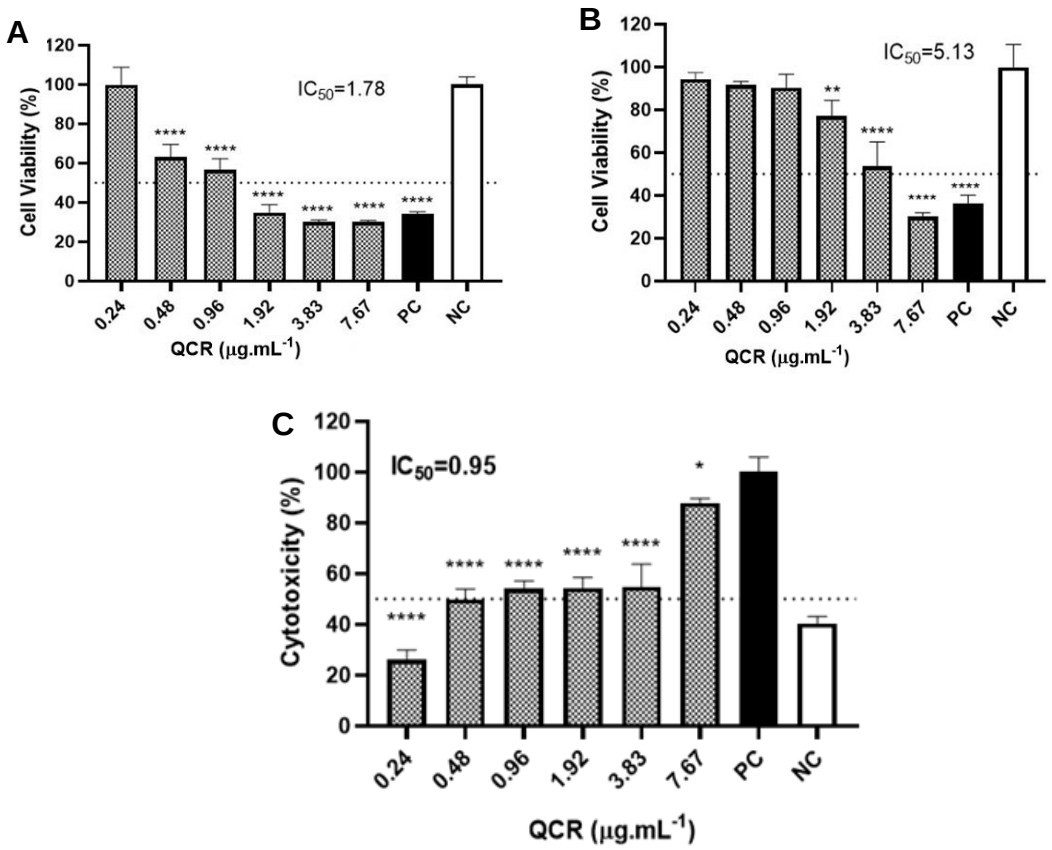

Figure 4. The cytotoxic effect of QCR on ZF-L cells was assessed using three different assays: (A) MTT assay, (B) NR uptake assay, and (C) LDH release assay. Cells were treated with QCR for 24 h at various concentrations. For (A) and (B), H_2O_2 was used as the positive control (PC). Data is presented as the percentage of mean ± SD, with the mean of the negative control (NC) set as 100%. In (C), Triton-100 X was used as the PC, with the PC set as 100%. To determine the significance of the data, a one-way ANOVA followed by Dunnett's post-hoc test was employed in comparison to the PC, where *p <0.05; **p <0.01; ***p <0.001 and ****p <0.0001.

Cells treated only with culture medium were used as the NC in all assays. 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. Three independent experiments were carried out in triplicate.

3.3.3. Lysosomal activity

Figure 4-B displays the results of lysosomal activity changes induced by the QCR herbicide formulation, assessed using the NR assay. ZF-L cells treated with QCR exhibited reduced lysosomal activity at concentrations of 0.48 to 7.67 µg/mL showing (36.69%, 43.36%, 65.01%, 69.72% and 69.83%, respectively) when compared to the control. The IC_{50} for this assay was 5.13 µg/mL.

3.3.4. LDH assay

Membrane damage was observed at concentrations of 0.96 to 7.67 µg/mL, with percentages of 54.05%, 54.34%, 54.80%, and 87.96%, respectively, compared to the positive control. The IC_{50} for this assay was 0.95 µg/mL (Figure 4-C).

3.3.5. Estimation of global IC_{50}

Table 1 shows the IC_{50} values for the different assays and the median global IC_{50}. The median global IC_{50} for the herbicide was determined to be 3.46 ± 1.68 µg/mL.

Table 1: Median global IC_{50} value of QCR calculated from the IC_{50} of the cytotoxicity tests

QCR	
Assay	IC_{50} (µg/mL)
Hemolytic assay	19.83
MTT assay	1.78
Neutral red assay	5.13
LDH assay	0.95
Median IC_{50}	3.46 ± 1.68

IC_{50}: The half-maximal inhibitory concentration

3.4. Biomarker dosages

Figure 5 shows the effects of QCR on biomarker dosing. Three concentrations were tested, based on the median IC_{50} values shown in Table 1: 1/2x (1.73 µg/mL), 1x (3.46 µg/mL), and 2x (6.92 µg/mL).

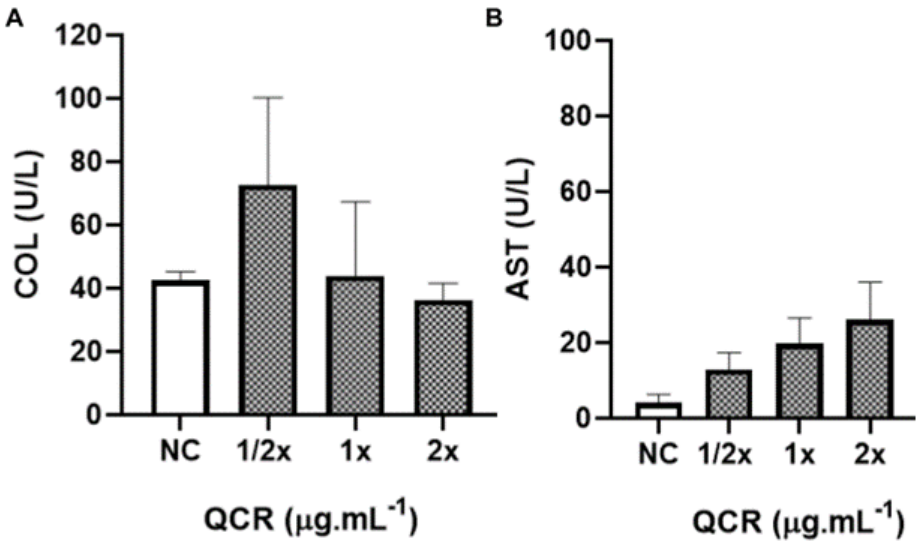

Figure 5. Biomarker dosages in ZF-L cells after 24 h of exposure to median IC_{50} concentrations: 1/2x (1.73 µg/mL); 1x (3.46 µg/mL) e 2x (6.92 µg/mL). (A) Cholinesterase (COL) activity (B) Aspartate aminotransferase (AST) activity. Untreated cells were used as the negative control (NC). Data is expressed as units of enzyme per liter (U/L). Samples were compared with the NC. Triplicate measurements were utilized to calculate the mean and standard deviation. Statistical analysis was conducted using a one-way ANOVA, followed by Dunnett's post-hoc test, with a significance level of $^{**}p < 0.01$ when compared to the negative control. Notably, no significant differences were observed for (A).

The results of the cholinesterase activity (Figure 5-A) reveal a concentration-dependent effect of QCR on the enzyme. At 1.73 µg/mL, cholinesterase activity was elevated to 70 U/L, suggesting a potential stimulatory effect of QCR at this lower concentration. This increase might indicate enhanced enzyme function or a reduced inhibition at this level. In contrast, at 3.46 µg/mL, cholinesterase activity returned to baseline levels (40 U/L), indicating that QCR at this concentration does not significantly alter enzyme activity. At 6.92 µg/mL, cholinesterase activity decreased to 30 U/L, suggesting an inhibitory effect of QCR at higher concentrations. Figure 5-B shows the impact of QCR on AST activity. The positive control demonstrated high AST levels (80 U/L), while the negative control showed low baseline activity. At 1.73

µg/mL, AST activity was slightly elevated (10 U/L) but remained close to the negative control. At 3.46 µg/mL, AST activity increased to 20 U/L, indicating a moderate effect of QCR. At 6.92 µg/mL, AST activity rose to 25 U/L, reflecting a further increase with higher QCR concentrations. These results suggest that QCR induces a dose-dependent increase in AST activity, although the effect is relatively modest compared to the positive control.

3.5. Oxidative stress effects on ZF-L cells

Figure 6 illustrates the changes in oxidative stress parameters in ZF-L cells following exposure to the QCR herbicide formulation. Three concentrations were tested, based on the median IC_{50} values shown in Table 1: 1/2x (1.73 µg/mL), 1x (3.46 µg/mL), and 2x (6.92 µg/mL).

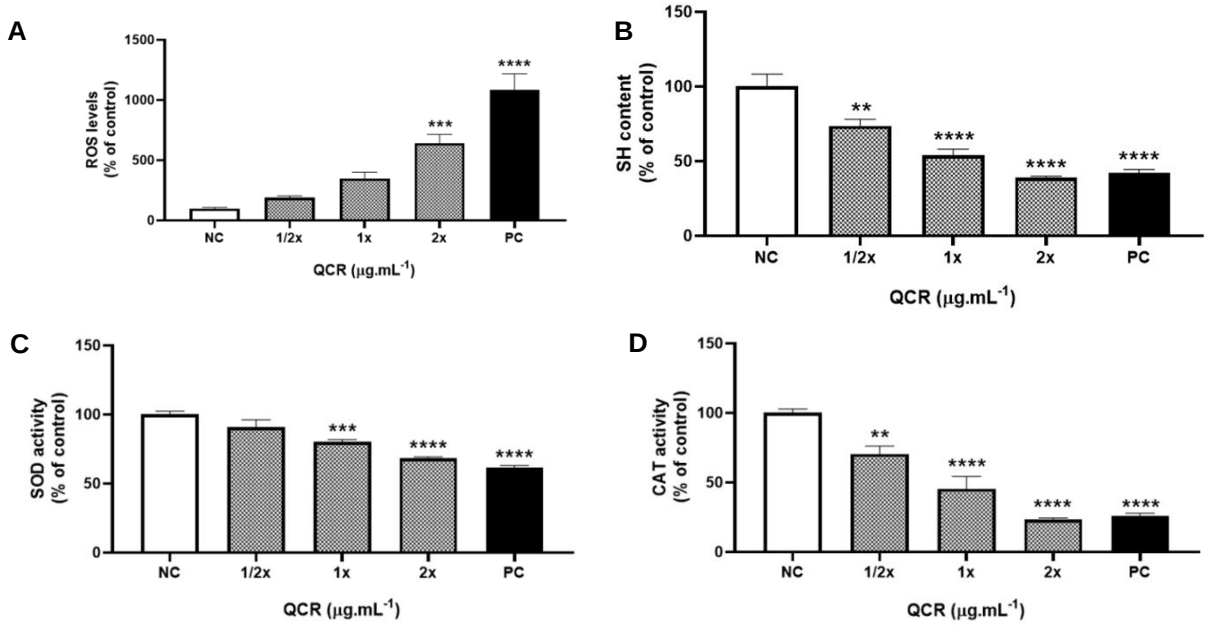

Figure 6. Oxidative stress parameters in ZF-L cells after 24 h of exposure to median IC_{50} concentrations: 1/2x (1.73 µg/mL); 1x (3.46 µg/mL) e 2x (6.92 µg/mL). (A) Reactive oxygen species (ROS); (B) Total sulfhydryl (SH) content; (C) Superoxide dismutase (SOD) activity; (D) Catalase (CAT) activity. Untreated cells were used as the negative control (NC). Data is expressed as a percentage of the NC value. ***p* <0.01; ****p* <0.001; *****p* <0.0001, compared with the NC. The positive control (PC) is also shown. Three independent experiments were carried out in duplicate.

The results revealed that QCR significantly increased ROS production by 542.31% at the 2x concentration (Figure 6-A). It also caused notable reductions in total sulfhydryl content at all tested concentrations, with decreases of 26.44%,

45.91%, and 61.04% for 1/2x, 1x, and 2x, respectively, compared to the negative control (Figure 6-B). Additionally, QCR reduced the activity of antioxidant enzymes SOD and CAT. At concentrations of 1x and 2x, SOD activity decreased by 19.99% and 31.69%, respectively, while CAT activity was significantly reduced by 29.44%, 59.50%, and 76.87% at 1/2x, 1x, and 2x, respectively (Figure 5-C, D).

4. Discussion

Smuggled agrochemicals, often unregistered with regulatory authorities, may have altered compositions and concentrations of active ingredients (Fraga et al., 2016; Crocoli et al., 2019; Berneira et al., 2020; Crocoli et al., 2020). Analysis of seized agrochemicals, including QCR, has revealed that these products often contain lower concentrations of active ingredients than those stated on their labels (Berneira et al., 2020). Most agrochemicals are soluble in water and persist in soil for extended periods, posing significant risks of contaminating both surface and groundwater sources and potentially harming non-target organisms (Coltro et al., 2017; Song et al., 2022). In this study, the presence of the active ingredient QCR was confirmed by GC-MS.

In the TGA, the QCR presented events of variations in temperature increases with mass losses at temperatures close to 100ºC that may be associated with water loss. In the DSC analysis, the physicochemical transitions occurred in endothermic peaks. However, as the components of the samples are unknown, it was not possible to identify what type of transition occurred in each event. Furthermore, agrochemicals are complex mixtures and often contain adjuvants in their formulations that are not always described by manufacturers (Mesnage et al., 2014; Mesnage and Antoniou, 2017; Leoci and Ruberti, 2021). In that regard, the thermal events presented may be related to other components that make up the herbicide formulation. Furthermore, it is important to consider that no data regarding TGA and DSC thermal analyses performed on an herbicide formulation containing QCR were found in the literature.

The HA assay, *in vitro*, has been used as an alternative method for assessment the cytotoxicity of several compounds, including agrochemicals (Farag and Alagawany, 2018). In this study, the hemolytic effect of QCR on sheep erythrocytes occurred at concentrations of 21.08 and 84.82 µg/mL, suggesting that QCR is cytotoxic and causes damage to erythrocytes membranes at these concentrations.

This result corroborates those described by Al-Alam et al. (2015), who demonstrated that the herbicide trifluralin presented HA at a concentration of 2.4 µg/mL. Furthermore, the fungicide mancozeb (100 µMol) also presented HA (Quds et al., 2023).

The cytotoxicity exerted by QCR was evaluated using the MTT, NR, and LDH release assays in ZF-L cells. It was observed that the herbicide reduced the activity of mitochondrial and lysosomal organelles. Furthermore, it also causes damage to the cell membrane. However, no data were found in the literature using QCR to compare these results. In relation to ZF-L cells exposed to other agrochemicals, Goulart et al. (2015) demonstrated that glyphosate-based agrochemicals reduced mitochondrial and lysosomal functionality of ZF-L cells after 6 h of herbicide exposure. Changes in these organelles were also observed by Lopes et al. (2018) after exposure to Roundup at a concentration of 3.25 µg/mL.

Regarding the effects of auxin herbicides on animal cells, Laborde et al. (2020) evaluated the cytotoxicity of 2,4-dichlorophenoxyacetic acid (2,4-D) and its commercial formulation Dedalo Elite, using the MTT and NR assays in Chinese hamster ovary cells (CHO-K1). The authors observed that Dedalo Elite was able to significantly reduce both mitochondrial and lysosomal activity in an exposure range of 4-100 µg/mL, while 2,4 D, only inhibited mitochondrial activity at concentrations of 10 and 100 µg/mL.

In the LDH release assay, loss of plasma membrane integrity was observed in ZF-L cells, indicating QCR cytotoxicity after 24 h of exposure to the herbicide, at concentrations of 0.96 to 7.67 µg/mL. A similar result was reported by Lovecka et al. (2015) who reported the cytotoxic effect on the in human liver carcinoma cells (HepG2), after 48 h of exposure to 100 µg/mL of the herbicides bromoxynil, chloroxynil, and ioxynil. The release of the LDH enzyme into the extracellular environment is related to cell membrane damage caused by some stressor (Lushchak et al., 2018; Huang et al., 2020).

Liver damage is a critical parameter to assess when evaluating the toxicity of agrochemicals, as it can indicate cellular injury or stress in response to these compounds (Nwonumara and Okogwu, 2020). In this context, the measurement of AST levels serves as a biomarker for hepatocellular damage and possible interferences with mitochondrial functions, as almost all AST is present in the mitochondria. In this study, exposure to the QCR herbicide led to a dose-dependent

increase in AST activity in ZF-L cells, indicating potential liver damage caused by this herbicide. Similar findings were reported by Abdelmagid et al. (2023), where elevated AST levels were observed in fish liver cells exposed to glyphosate, a widely used herbicide. Furthermore, Gök et al. (2022) demonstrated increased AST activity in response to pesticide exposure in rodent liver tissues, suggesting hepatotoxicity. The significant elevation in AST observed in this study, particularly at higher concentrations of QCR, aligns with these findings and underscores the hepatotoxic potential of QCR.

The modulation of cholinesterase (COL) activity in ZF-L cells upon exposure to the QCR herbicide suggests specific hepatocellular responses to this agrochemical. Cholinesterase enzymes in hepatocytes are crucial for maintaining cellular homeostasis, particularly in the metabolism of various substances, including drugs and toxins (Benitez et al., 2021). In this study, the exposure to sub-lethal concentrations of QCR led to a significant increase in COL activity, indicating a possible hepatocellular adaptive response to the initial chemical stress. This increase in enzyme activity might reflect the activation of detoxification pathways within the liver cells, where COL could play a role in neutralizing or processing the herbicide or its metabolites (Hernández et al., 2013). However, at higher concentrations the reduction in COL activity suggests that higher doses of QCR may surpass the hepatocytes' adaptive capacity, leading to enzyme inhibition. (Manfo et al., 2021). The parallel increase in AST levels supports this hypothesis, as elevated AST is a well-known marker of hepatocellular injury and stress.

Oxidative stress is an important parameter to evaluate the effect of agrochemicals on cells, as it can indicate the disorder caused by the overproduction of ROS (Kaur, 2019) and the alteration in the action of antioxidant defense mechanisms, including non-enzymatic (thiol groups) and enzymatic (SOD/CAT) systems (Badgujar et al., 2015). Regarding the evaluation of oxidative stress parameters, the QCR herbicide increased the production of ROS and decreased the response of the antioxidant system in ZF-L cells. Increased ROS levels in ZF-L cells exposed to metal-insecticide-hesperidin (MgHP) (1 µg/mL) after 24 h were described by Bonomo et al. (2020). Furthermore, Xie et al. (2022), observed that thiacloprid increased the generation of ROS in ZF-L cells cultured with 100 to 800 µM of the insecticide after 24 h of exposure and Huang et al. (2020) reported the production of ROS induced by the herbicide acetochlor (400 mM) in HepG2 cell line. In addition, in

this study, the results indicated that the exposure of ZF-L cells to QCR had a significant decrease in the action of the antioxidant defenses total SH content and in the CAT and SOD. The results obtained in this study are in line with Bonomo et al. (2020), where an increase in ROS levels and a decrease in CAT after exposure of ZF-L cells to the insecticide MgHP was observed.

5. Conclusion

The current study highlights several critical findings regarding the seized QCR-containing formulation. Notably, the herbicide demonstrated cytotoxic effects *in vitro*, impacting both red blood cells and the ZF-L cell line across various concentrations and conditions. Additionally, oxidative stress was evident in ZF-L cells, as indicated by increased ROS levels and diminished antioxidant defenses. The study also revealed significant alterations in liver enzyme activities: an initial rise followed by a pronounced decline in COL activity and a dose-dependent increase in AST levels, which underscore the herbicide's possible hepatotoxicity. These observations show the utility of *in vitro* assays in assessing the cytotoxicity of seized pesticides, emphasizing their potential to harm non-target organisms, particularly concerning liver function and broader metabolic disturbances.

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5 Discussão

O Brasil é uma referência mundial quanto ao elevado consumo de agroquímicos, incluindo herbicidas e, também tem se destacado no cenário nacional, quanto à utilização destes produtos oriundos de contrabando (GABOARDI; CANDIOTTO; PANIS, 2023), resultando em muitas apreensões, pela Polícia Federal, em várias regiões do país (DE MORAES, 2022). A utilização dessas formulações, além de trazer implicações econômicas, pode representar riscos à produção agrícola, às pessoas, seja de forma direta ou indiretamente, ao meio ambiente e a outras espécies não-alvos, incluindo organismos aquáticos (MALKOV; PRISCHEPA; KUTONOVA, 2015; FREZAL; GARSOUS, 2020).

Os princípios ativos das formulações dos herbicidas IMZT e QCR utilizados neste estudo foram confirmados por CG-EM, corroborando os achados de Berneira *et al*. (2020). Estes mesmos autores demonstraram que a concentração do ingrediente ativo de cada formulação estava bem abaixo do valor que constava no respectivo rótulo. Cabe ressaltar que tanto o IMZT quanto o QCR são agroquímicos permitidos pela legislação brasileira para uso na produção agrícola; porém, as amostras utilizadas neste estudo são oriundas do comércio ilegal e apreendidas pela Polícia Federal (BERNEIRA *et al*., 2020).

Na análise termogravimética (TGA), ambas formulações apresentaram eventos de variações de aumentos de temperatura com perdas de massas. Considerando que agroquímicos, em sua maioria, são misturas complexas, possuem em sua formulação aditivos, nem sempre divulgados pelos fabricantes (VAN DE MERWE *et al*., 2018; BAYOUMI, 2021), muitos destes eventos podem estar relacionados a quaisquer outros ingredientes que compõem os herbicidas estudados. Contudo, não foram encontrados dados na literatura para comparação dos resultados aqui reportados

Na análise de DSC tanto para o IMZT quanto para o QCR, as transições físico-químicas ocorreram em picos endotérmicos. No entanto, como os demais componentes das amostras são desconhecidos, não há como afirmar que tipo de transição ocorreu em cada evento. Como reportado acima para TGA, não foram observados dados referentes a DSC para o IMZT e QCR até o presente momento, assim reportamos primeiramente nesse estudo.

Neste trabalho, demonstramos que as formulações apreendidas dos herbicidas IMZT e QCR produziram citotoxicidade em eritrócitos de mamífero e em diferentes mecanismos celulares e alterações em parâmetros de estresse oxidativo na linhagem celular ZF-L.

No ensaio hemolítico, foi revelado que tanto o IMZT quanto o QCR foram citotóxicos para as hemácias nas concentrações mais elevadas de 19,29 a 308,64 µg/mL e 21,08 e 84,82 µg/mL, respectivamente. Após 1 hora de incubação, os valores de IC_{50} foram de 12,75 µg/mL (IMZT) e de 19,83 µg/mL (QCR). A hemólise causada pela exposição a agroquímicos pode ser o resultado de lesão direta e/ou indireta às células (AHMAD; AHMAD; 2017), pois o sangue representa um dos principais alvos dos xenobióticos quando entram no organismo, e as hemácias, as células mais abundantes no sangue, são especialmente suscetíveis aos efeitos nocivos dos agroquímicos (SALAM; ARIF; MAHMOOD, 2020). Os resultados obtidos em nosso estudo corroboram com os de outros pesquisadores que evidenciaram danos na membrana dos eritrócitos, *in vitro*, durante o tratamento com agroquímicos devido ao aumento da lise celular (AHMAD; AHMAD, 2017; SALAM; ARIF; MAHMOOD, 2020; QUDS *et al*., 2023). Este é o primeiro estudo a demonstrar os efeitos de formulações apreendidas de agroquímicos contendo IMZT e QCR pelo ensaio de citotoxicidade em eritrócitos.

Na sequência, avaliamos a citotoxicidade em células ZF-L, expostas aos herbicidas IMZT e QCR, nas concentrações de, aproximadamente, 10x menores das utilizadas para o ensaio hemolítico. Após o período de exposição (24 horas), observamos que ambos herbicidas diminuíram a atividade mitocondrial e causaram danos aos lisossomos e danos à integridade da membrana celular, nas concentrações mais elevadas. As IC_{50} do IMZT foram de 3,01, 2,67 e 1,61 µg/mL e para o QCR foram de 1,78, 5,13 e 0,95 µg/mL, respectivamente.

A integridade das enzimas desidrogenases mitocondriais reflete, indiretamente, a respiração celular mitocondrial e geralmente está associada à atividade da enzima succinato desidrogenase (MESNAGE *et al*., 2014; TAJU *et al*., 2017; NAGY *et al*, 2020). No entanto, na avaliação da atividade mitocondrial pelo ensaio MTT, quando ocorre uma redução desta atividade, também pode ser um indicativo de falha nos processos energéticos, o que pode levar a um comprometimento na energia necessária para manter a integridade do metabolismo celular (LOPES; SANDRINI; SOUZA, 2018; BONOMO *et al*., 2019). Respostas

semelhantes também podem ser consideradas ao estimar a capacidade de retenção do vermelho neutro. A quantidade de corante retida pelos lisossomos, geralmente, é proporcional ao número de células viáveis (KOLAROVA; VELISEK; SVOBODOVA, 2021). Além disso, a liberação da enzima LDH ao meio extracelular devido a danos à membrana plasmática é um indicativo de morte celular irreversível e está associada à indução do estresse oxidativo nas células (SEVIM *et al*., 2020).

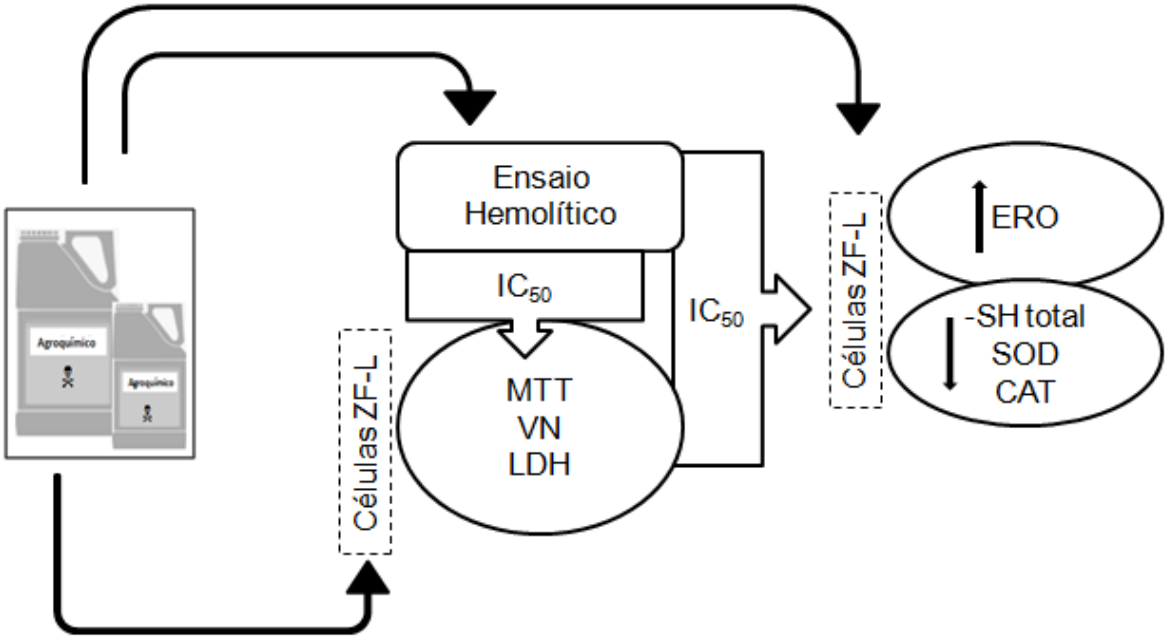
Também avaliamos a ocorrência de estresse oxidativo em células ZF-L expostas, por um período de 24 horas, aos herbicidas IMZT e QCR, nas concentrações de 1/2x, 1x e 2x, correspondentes à mediana de suas IC_{50} dos ensaios de citotoxicidade. Foram avaliados parâmetros oxidativos como a formação de ERO e as atividades antioxidantes do teor de sulfidrila total e das enzimas SOD e CAT. O aumento de ERO observado foi acompanhado por uma redução na atividade das enzimas antioxidantes e do conteúdo sulfidrílico (tiólico) total, sendo semelhantes nas concentrações de 2x, para ambos herbicidas (5,68 µg/mL e 6,92 µg/mL, respectivamente). Estas alterações são indicativas de estresse oxidativo e respostas antioxidantes.

O estresse oxidativo induzido por agroquímicos tem sido o foco de muitas pesquisas na última década, como um possível mecanismo de toxicidade (CORTÉS-IZA; RODRÍGUEZ, 2018; SEVIM *et al*., 2020). A produção anormal de ERO pode resultar em danos significativos à estrutura celular, sendo considerado um sinal importante de dano oxidativo (SANTANA *et al*., 2022). A partir dos sistemas endógenos de proteção contra os efeitos nocivos a elevados níveis de ERO, peixes, assim como muitos outros organismos, podem combater as ERO com a produção de enzimas antioxidantes como a SOD e a CAT, que eliminam formação das espécies reativas $O_2^{\cdot -}$ e do H_2O_2, pela conversão em espécies menos reativas (SANTANA *et al*., 2022; MARTINS *et al*., 2024). Além disso, o grupo tiólico, juntamente com fatores não enzimáticos e enzimáticos, regula o metabolismo intracelular, defendendo as estruturas e funções biológicas do ataque nocivo das ERO (KÜKÜRT *et al*., 2021). Portanto, baixos níveis do conteúdo tiólico total podem comprometer a função proteica em vários grupos importantes, como as enzimas, que geralmente sofrem danos devido ao aumento de ERO (JABŁOŃSKA-TRYPUĆ, 2017; PEDRA *et al*., 2018).

Todavia, como demonstrado no nosso estudo, quando ocorre uma diminuição significativa nas atividades de SOD e CAT, bem como do conteúdo tiólico total,

associada a elevados níveis de ERO, pode-se presumir que ocorreu uma falha da linha de defesa antioxidante (SHUKLA *et al*., 2017; XIE *et al*., 2022). Diversos estudos apontam desordens nos parâmetros de estresse oxidativo causados pela exposição a formulações de herbicidas contendo IMZT ou QCR em vários organismos vertebrados não-alvos, incluindo anfíbios (DORNELLES; OLIVEIRA, 2016; COLTRO *et al*., 2017; PÉREZ-IGLESIAS *et al*., 2023; REICHERT *et al*., 2022), peixes (MORAES *et al*., 2011; PRETTO *et al*. 2011; TONI *et al*., 2013; MENEZES *et al*. 2012; MENEZES *et al*., 2014; SALEH *et al*., 2022) e mamíferos (YAO *et al*., 2020). No entanto, não foram encontrados relatos referentes à indução de estresse oxidativo em células ZF-L, para estudo comparativo. Na Figura 6, estão compilados os principais resutados observados neste estudo.

Figura 6 **–** Esquema dos principais resultados obtidos no estudo.



Fonte: O autor.

6 Conclusões

- Os ingredientes ativos das formulações dos herbicidas IMZT e QCR foram confirmados por CG-EM e as caracterizações térmicas das formulações dos herbicidas foram realizadas;

- As formulações dos herbicidas contendo IMZT ou QCR, nas condições e concentrações testadas, *in vitro*, induziram citotoxicidade em eritrótrocitos de mamífero e em hepatócitos de *D. rerio* (linhagem ZF-L);

- As formulações dos herbicidas contendo IMZT ou QCR, nas condições e concentrações testadas, *in vitro*, foram capazes de induzir o estresse oxidativo em hepatócitos de *D. rerio* (linhagem ZF-L), confirmado pelo aumento das ERO e pela diminuição do sistema de defesa antioxidante avaliado.

- Esses resultados revelam a importância de ensaios *in vitro* para a avaliação da toxicidade de agroquímicos apreendidos, pois demonstram os efeitos nocivos que podem afetar a saúde humana e o meio ambiente.

7 Perspectivas

A partir das IC_{50} determinadas para o IMZT e QCR é possível aprimorar os estudos, a partir dos seguintes objetivos:

- Realizar a detecção molecular dos níveis de expressão gênica de alguns genes relacionados ao extresse oxidativo e do sistema ABC, por exemplo, ABCB1 e ABCC1, em células ZF-L;

- Avaliar a genotoxicidade pela técnica do PicoGreen® e pelo ensaio cometa;

- Realizar ensaios *in vivo* em larvas e em diferentes tecidos do peixe-zebra exposto aos herbicidas;

- Estudar a toxicidade *in vivo* utilizando modelos alternativos de ecotoxicidade.

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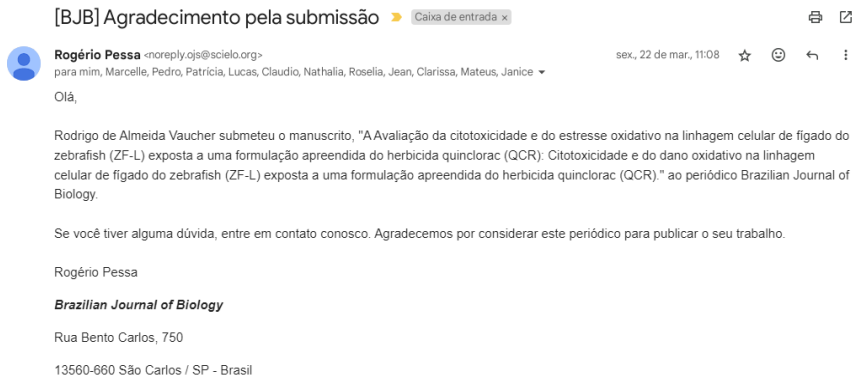
ANEXO

ANEXO – Comprovante de submissão



[BJB] Agradecimento pela submissão Caixa de entrada ×

Rogério Pessa <noreply.ojs@scielo.org> sex., 22 de mar., 11:08

para mim, Marcelle, Pedro, Patrícia, Lucas, Claudio, Nathalia, Roselia, Jean, Clarissa, Mateus, Janice

Olá,

Rodrigo de Almeida Vaucher submeteu o manuscrito, "A Avaliação da citotoxicidade e do estresse oxidativo na linhagem celular de fígado do zebrafish (ZF-L) exposta a uma formulação apreendida do herbicida quinclorac (QCR): Citotoxicidade e do dano oxidativo na linhagem celular de fígado do zebrafish (ZF-L) exposta a uma formulação apreendida do herbicida quinclorac (QCR)." ao periódico Brazilian Journal of Biology.

Se você tiver alguma dúvida, entre em contato conosco. Agradecemos por considerar este periódico para publicar o seu trabalho.

Rogério Pessa

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